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DISODIUM PHOSPHATE AS A CATALYST FOR THE QUANTITATIVE OXIDATION OF GLUCOSE TO CARBON DIOXIDE WITH HYDROGEN PEROXIDE.

By EDGAR J. WITZEMANN.

(From the Otho S. A. Sprague Memorial Institute, Rush Medical College, Chicago.)

(Received for publication, July 13, 1920.)

The experiments described in this paper represent a confirmation and extension of part of Löb's observations on the influence of phosphates on oxidative glycolysis. By the experiments herein described it is proved that disodium phosphate catalyzes the quantitative oxidation of glucose to carbon dioxide by hydrogen peroxide. Additional experiments on the influence of the carbonates of sodium and other compounds are included and a partial interpretation of the results is offered.

Previous experiments on the influence of phosphates on the oxidation of butyric acid[1] with hydrogen peroxide were being extended by further experiments when it was realized that if the results of Löb and his coworkers,[2, 3] on the influence of phosphates on glucose oxidation with peroxide, could be demonstrated by an adequate method the results would help clarify the influence of phosphates on butyric acid oxidation and have considerable interest in other ways. The results of some earlier work on the oxidation of glucose[4] indicated that probably the amount of oxidation observed by Löb could be exactly determined by the method suggested by those data. This was confirmed.

[1] Witzemann, E. J., *J. Biol. Chem.*, 1918, xxxv, 83.

[2] Löb, W., and Pulvermacher, G., *Biochem. Z.*, 1910, xxix, 316. Löb, W., and Gutmann, S., *Biochem Z.*, 1912, xlvi, 288. Beysel, W., and Löb, W., *Biochem. Z.*, 1915, lxviii, 368.

[3] Löb, W., *Biochem. Z.*, 1911, xxxii, 43.

[4] Witzemann, E. J., *J. Am. Chem. Soc.*, 1916, xxxviii, 150.

The statements under consideration as given in Löb's summary[3] are quite definite and are in part as follows:

"(1) In salt-free sugar solutions hydrogen peroxide produces only a vanishingly small amount of oxidative glycolysis.

(2) The glycolysis is markedly increased by raising the hydroxyl ion concentration.

(3) With the small OH ion concentrations in solutions having the alkalinity of blood, which is only slightly different from that of water, glycolysis is very slight if it is not accelerated by phosphates.

(4) The phosphate ions accelerate the glycolysis by the OH ions; the most favorable OH ion concentration within the limits tested lies at pH 8.302 to 7.070. At pH $\overline{<}$ about 5.600 there is no longer a perceptible OH ion effect exceeding that of pure water, even in the presence of phosphate ions.

(5) The acceleration of the glycolysis increases with constant OH ion concentration with increase in the absolute amount of phosphate added."

An examination of the experimental data, however, leaves one in doubt as to whether Löb really measured the oxidative glycolysis. In fact Michaelis and Rona[5] were not convinced by Löb's data and interpreted his observations differently. Obviously his determination of optical rotation and the reduction of Fehling solution by the glucose solutions before and after oxidation was not a determination of the absolute amount of oxidation. Consequently the term "oxidative glycolysis," which he uses to describe these phenomena, might include two processes.

(*a*) Destruction of glucose by oxidation at the expense of oxygen from the hydrogen peroxide used. This is what Löb meant.

(*b*) Destruction of glucose by intramolecular rearrangement under the influence of alkali. This kind of chemical change is what Michaelis and Rona appear to think Löb really saw at least in part.

If a neutral phosphate system such as Löb used, which is known to be a constituent of many living organisms, has any considerable effect upon oxidation the scope and nature of the effects should be known. The possible importance of such facts biologically for instance, when considered in relation to the well known indispensible relationships between phosphates and much normal cellular oxidation, is too obvious to require further comment.

[5] Michaelis, L., and Rona, P., *Biochem. Z.*, 1912, xlvii, 447.

The data described in this paper are sufficiently definite to give a new interest to the many facts already in hand in this field and to serve as a definite point of reference in the further study of these questions.

The results described here have a general interest in another way also. In the interpretation of the action of alkaline substances on sugars two points of view are recognized. According to the one the known effects of alkaline substances on sugars are due essentially to the hydroxyl ions. The other older view recognizes that the undissociated molecules and other ions may also aid or produce other effects than those of the so called hydroxyl ion effects. Without reviewing this problem any further in this paper it may simply be stated that the data herein presented offer varied and interesting support for the latter view.

EXPERIMENTAL.

Considering the importance of Löb's claims from several points of view it seemed highly important to determine accurately how much oxidation actually took place in his experiments with phosphates. This, it was thought, could be done, by applying the results of the author's previous study of the complete oxidation of glucose with potassium permanganate[4] to the analysis of the results obtained by Löb's experiments.

1. Methods of Analysis.—Previous experiments on the oxidation of glucose showed that in alkaline solution it is quantitatively oxidized to carbon dioxide and oxalic acid with potassium permanganate. The oxalic acid in turn is quantitatively oxidized to carbon dioxide by permanganate in sulfuric acid solutions. The plan was therefore as follows:

1. Oxidize glucose with hydrogen peroxide in the presence of phosphates just as Löb did.

2. After the expiration of the proper time interval add excess manganese dioxide to decompose unchanged hydrogen peroxide.

3. After decomposition is complete filter off the manganese dioxide, washing the filter and the original flask thoroughly.

4. Add excess sodium hydroxide solution.

5. Add an accurately known amount but excess of a strong accurately standardized solution of potassium permanganate

(about 3 gm. per 100 cc.). Heat this mixture to boiling and set aside over night after covering the top of the hot flask.

6. Add excess concentrated sulfuric acid.

7. Add an accurately known amount but excess of an accurately standardized solution (about 6 gm. per 100 cc.) of oxalic acid.

8. Dilute the clear colorless solution to a convenient definite volume and using an aliquot portion titrate back the excess oxalic acid with dilute potassium permanganate solution (0.1 N).

9. Calculate the total permanganate required for complete oxidation of the solution in No. 8, add the permanganate added in the beginning, and substract the permanganate equivalent of the oxalic acid used. The result is the amount of permanganate utilized by the glucose or other incompletely oxidized compounds present and may easily be calculated to its glucose equivalent.

In order to test the accuracy of the above method a solution of pure glucose containing 10 gm. per liter was prepared. 20 cc. of this solution, containing 0.200 gm. of glucose, 15 cc. of 35 per cent sodium hydroxide solution, and 75 cc. (= 2.028 gm.) of potassium permanganate solution were heated to boiling. After standing over night excess concentrated sulfuric acid was added and then 50 cc. (≡ 1.519 gm. of $KMnO_4$) of an oxalic acid solution. This colorless solution was diluted to 500 cc. in a graduated flask. 25 cc. portions were titrated back with 0.0996 N potassium permanganate. 5.70 cc. were required. $5.70 \times 20 \times 0.003146 = 0.359$ gm. of $KMnO_4$ required for the excess oxalic acid that was added.

2.028	gm.	$KMnO_4$ originally added.
0.359	"	$KMnO_4$ required for excess oxalic acid.
2.387	"	$KMnO_4$ used (total).
1.519	"	$KMnO_4$ equivalent of oxalic acid added.
0.868	"	$KMnO_4$ reduced by the glucose.

Since 2.40 molecules of $KMnO_4$ are required to oxidize 1 molecule of glucose to carbon dioxide the equation

$$758.4 : 180 = 0.868 : x$$
$$x = 0.206 \text{ gm. glucose}$$

gives the amount of glucose originally present.

Another oxidation made at the same time gave 0.198 gm. of glucose.

These were the results obtained with the first pair of oxidations tried and give fairly the maximum analytical error as demonstrated by subsequent experience. The results show that the method will be satisfactory provided the amount of oxidation observed exceeds the experimental error of 2 or 3 per cent.[6]

Experiments with the Phosphates of Sodium.

2. Repetition of Löb's Experiments.—Having established the fact that it is possible to determine glucose quantitatively in the proposed way the author repeated and analyzed by the method described above a number of the experiments carried out by Löb.

In Table I the results obtained in five oxidations carried out at room temperature for just 1 week are given. The results are calculated as though the oxygen required by the unoxidized compounds in the solution was all consumed by unchanged glucose. This is almost certainly not entirely true but since the incompletely oxidized compounds are possibly a complex mixture, difficult to analyze,[7] it seemed permissible and correct for the purposes of comparison to calculate the permanganate consumed to glucose. The results show that the influence of the phosphates

[6] A similar method was developed by Greifenhagen and coworkers (Greifenhagen, W., König, J., and Scholl, A., *Biochem. Z.*, 1911, xxxv, 169), and was found sufficiently accurate in use by Levene and Meyer (Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 265). These results were discovered after the completion of my own work.

[7] On the basis of Löb's earlier work (Löb, W., *Biochem. Z.*, 1908, xii, 78, 466; 1909, xvii, 132. Löb, W., and Pulvermacher, G., *Biochem. Z.*, 1909, xvii, 343. Löb, W., *Biochem. Z.*, 1909, xx, 516; xxii, 103; 1910, xxiii, 10; xxvi, 231), but specifically on the basis of a later statement (Löb, W., *Biochem. Z.*, 1915, lxviii, 368) it might be concluded that the incompletely oxidized compounds are formic and polyhydroxy acids arising from formaldehyde and pentoses. Tests on solutions from complete oxidations known to reduce permanganate equivalent to 0.02 to 0.04 gm. glucose in 75 cc. gave distinct tests for sugar with Haines' or Fehling's solution. Since this is near the limits of sensitiveness of these reagents, it appears that no large proportion of intermediate oxidation products (between hexose and CO_2) can be present. This appears to conform with the observations of Smolka (Smolka, A., *Sitzungsb. Math. Natur. Akad. Wiss.*, 1887, xcv, pt. ii, 5) on the oxidation of glucose with insufficient neutral permanganate, who recovered only final oxidation products (HCO_2H, $H_2C_2O_4$, and CO_2) and the calculated amount of unchanged glucose.

is progressively greater with increasing concentration, but that it is not a linear function of the concentration since the relative acceleration diminishes with increasing phosphate concentration.

The results in Table II were obtained under exactly the same conditions as those in Table I except that the solutions were kept 98 hours (4 days, 2 hours) in an incubator at 37°C.

TABLE I.

Glucose + H_2O_2 + Phosphates at Room Temperature.

20 cc. glucose solution (0.200 gm.) + 20 cc. 3 per cent H_2O_2 in total volume of 75 cc.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Reaction.	Glucose recovered.	Glucose oxidized.
	cc.	*cc.*	*cc.*	*pH*	*gm.*	*per cent*
1	0.0	0.0	35	7.07	0.2006	0.00
2	1.6	0.4	33	7.347	0.1863	6.85
3	6.4	1.6	27	7.347	0.1210	39.50
4	16.0	4.0	15	7.347	0.0658	67.10
5	25.6	6.4	3	7.347	0.0442	77.90

TABLE II.

Glucose + H_2O_2 + Phosphates at 37°.

20 cc. glucose solution (0.200 gm.) + 20 cc. 3 per cent H_2O_2 in total volume of 75 cc.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Reaction.	Glucose recovered.	Glucose oxidized.
	cc.	*cc.*	*cc.*	*pH*	*gm.*	*per cent*
1	0.0	0.0	35	7.07	0.186	6.50
2	1.6	0.4	33	7.347	0.1163	41.85
3	6.4	1.6	27	7.347	0.019	90.50*
4	16.0	4.0	15	7.347	0.034	83.00
5	25.6	6.4	3	7.347	0.035	82.50

* In the experiments at 37° it was generally observed that oxidation was less complete in No. 5 than in Nos. 3 or 4. Special experiments to interpret this apparent anomaly have not been done but the effect appears to be due to the fact that the velocity of oxygen activation by the Na_2HPO_4 is greater than the velocity of oxygen consumption and consequently the excess active oxygen is lost as such from the reaction mixture. This interpretation is so far supported by facts given in this paper and by others not mentioned.

3. Fate of the Glucose.—The results given above prove quite conclusively that the glucose is oxidized. Of the large number of compounds into which it could conceivably be converted without oxidation only a small number are not completely oxidizable to carbon dioxide by permanganate in acid or alkaline solution.[8] Nevertheless it seemed necessary to demonstrate actually that carbon dioxide was an important product of this oxidation.

All the experiments on carbon dioxide recovery were done with mixtures corresponding to No. 5 in Tables I and II. In determining the CO_2 the oxidation mixture was placed in a round bottom flask attached to a reflux condenser and arranged so that CO_2-free air could be bubbled through the mixture and then passed through wash bottles containing clear barium hydroxide solution.[9] On warming the flask nearly all the CO_2 was driven over. Excess dilute sulfuric acid was finally added and the mixture heated to boiling.

A. 75 cc. of such a solution, which had been kept in the incubator until all peroxide was gone and in which oxidation was nearly complete, gave in the CO_2 apparatus 0.13 gm. of barium carbonate or about 10 per cent of the calculated CO_2 yield. The rest of the CO_2 had been lost into the air.

B. 75 cc. of such a solution after 10 days at room temperature gave 0.69 gm. of $BaCO_3$, equivalent to 0.154 gm. of CO_2 or a 52.6 per cent yield of CO_2. The solution, to which excess sulfuric acid had been added while in the CO_2 apparatus, was alkalinized with sodium hydroxide, treated with MnO_2 to remove unchanged peroxide, filtered, and treated as usual with permanganate. The permanganate consumed was equivalent to 0.0749 gm. of glucose or 37.5 per cent recovered. $52.6 + 37.5 = 90.1$ per cent of the 0.200 gm. of glucose used recovered in this way.

Results similar to this were obtained under the same conditions a number of times.

[8] It was not until the experiments described above had been completed that it was suspected that oxidation to CO_2 was nearly quantitative. Löb expressed the opinion that formic and polyhydroxy acids are the main products and there was no reason to doubt this until the small amount of permanganate required to complete the oxidation suggested that the oxidation might already be largely completed to CO_2.

[9] Evans, W. L., and Witzemann, E. J., *J. Am. Chem. Soc.*, 1912, xxxiv, 1086.

C. In order to obtain a more complete conversion into CO_2 and a good recovery the oxidation was set up in the incubator. Two strong round bottom 300 cc. flasks, one of which contained barium hydroxide solution and the other the glucose oxidation mixture, were connected by a glass tube having two right angle bends in rubber stoppers. The stoppers were wired in and then covered over with molten paraffin. The whole was placed in the incubator at 37°C. and agitated a few moments every day for a week. It was then taken out and allowed to stand at room temperature several days with occasional agitation. The oxidation mixture gave 0.28 gm. of $BaCO_3$ in the CO_2 apparatus. The attached $Ba(OH)_2$ flask gave 0.83 gm. of $BaCO_3$. This corresponds to 0.2474 gm. of CO_2 altogether or an 84.4 per cent yield of CO_2. The oxidation mixture treated as in (B) reduced permanganate equivalent to 0.0195 gm. of glucose or 9.8 per cent of the glucose used. $84.4 + 9.8 = 94.2$ per cent of the glucose recovered in this way.

On the basis of these results there can be no doubt that the glucose unaccounted for by the permanganate consumed is really oxidized to CO_2.

In developing the above proof that practically quantitative oxidation to CO_2 is obtained several other facts were observed.

1. The carbon dioxide formed is freely and easily lost from the solution during the oxidation even at the room temperature. In this the oxidation resembles vital oxidation in which the carbon dioxide is spontaneously lost during respiration. As much as two-thirds or more of the carbon dioxide obtained is evolved and absorbed by barium hydroxide in a closed apparatus at 37°C.

Ordinary alkaline oxidation systems, although they undergo changes in many ways similar to those occurring in living organisms, differ in that the CO_2 formed is bound and held in the system as carbonate or bicarbonate. This easy formation and loss of CO_2 is probably the most important physical characteristic of a vital oxidation system. It is not yet certain to what extent the phosphate systems can carry out the other functions belonging to alkaline systems, that are so important in the non-oxidative transformations of sugar in organisms, but indications are not lacking that they can also aid in some of these changes under suitable conditions.

2. The solutions in which all hydrogen peroxide had disappeared and which contained material oxidizable by permanganate equivalent to only 0.02 to 0.04 gm. of glucose in 75 cc. (*i.e.*, 0.027 to 0.053 per cent) reduced Fehling solution distinctly. Since this is close to the limits of sensitiveness of this test with pure glucose it is clear that most of the glucose attacked had been completely burned to carbon dioxide, and that no appreciable quantity of intermediate products such as polyhydroxy acids could be present.

4. Influence of Additional Glucose and Peroxide.—On the basis of the results in the preceding section it was of considerable interest to know whether the same phosphate mixture would repeatedly catalyze the oxidation of glucose. In other words whether the products of the reaction in any way "poison" the catalyst. If CO_2 is the sole final product and if it is evolved as was shown above, the phosphate mixture should serve repeatedly in this oxidation just as it is known to do in the fermentation of glucose.[19]

Experiment 5, Table II, was set up in the incubator. After 3 days it was free from peroxide. 0.20 gm. of glucose and 20 cc. of 3 per cent peroxide were again added. After 1 week in the incubator the peroxide had again disappeared. The same materials were again added. After another week this was repeated. On determining the permanganate consumed in the usual way it was found to correspond to 0.0831 gm. of glucose. Since 0.80 gm. of glucose had been used this corresponds to 10.4 per cent of the glucose used, which is about what is recovered from a single experiment of this kind.

These results show that the functional activity of the disodium phosphate is not impaired in the catalysis. Since this does not occur it is clear that the disodium salt is not changed into monosodium phosphate by the carbonic acid, nor any other acid intermediate oxidation product, to any marked extent. If sodium bicarbonate were formed in this way the oxidation would be retarded or stopped in the typical way in which this compound acts (*cf.* Section 7).

[19] Harden, A., and Young, W. J., *J. Chem. Soc.*, 1905, xxi, 189; *Proc. Roy. Soc. London, Series B*, 1906, lxxvii, 405; 1908, lxxx, 299; 1909, lxxxi, 336. Young, W. J., *Proc. Roy. Soc. London, Series B*, 1909, lxxxi, 529. Harden, A., and Young, W. J., *Biochem. Z.*, 1911, xxxii, 173. Young, W. J., *Biochem. Z.*, 1911, xxxii, 177.

5. Influence of Changing the Ratio of the Phosphates.—The results in Table III constitute a repetition of part of Löb's experiments (Table XI)[3] on the influence of a change in the ratio of the two phosphates. All the experiments were set up in 250 cc. Florence flasks and kept in the incubator for 45 hours before analyzing.

TABLE III.

25 cc. (0.25 gm.) glucose + 25 cc. H_2O_2 + 20 cc. salt solution + 5 cc. water at 37°.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Reaction.	Glucose recovered.	Glucose oxidized.
	cc.	*cc.*	*cc.*	*pH*	*gm.*	*per cent*
1	0	0	25	7.07	0.2407	3.7
2	16	4	5	7.347	0.1992	20.3
3	10	10	5	6.813	0.2068	17.3
4	4	16	5	6.239	0.2316	7.4
5	2	18	5	5.910	0.2342	6.3

TABLE IV.*

20 cc. glucose (0.200 gm.) + 20 cc. 3 per cent H_2O_2 at 37° for 10 days.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Glucose recovered.	Glucose recovered after 2 days.
	cc.	*cc.*	*cc.*	*gm.*	*gm.*
1	25.6	6.4	59	0.0242	0.0387
2	25.6	32.0	34	0.0180	0.0271
3	25.6	64.0	2	0.0166	

* All the experiments in this table have a total volume of 131 cc. The reaction of No. 1 is distinctly alkaline to litmus paper while that of No. 3 is distinctly acid. Accordingly pH passes from a point on the alkaline side (about 7.347) to a point decidedly on the opposite or acid side of neutrality.

The results show a diminishing velocity of glucose oxidation as the ratio of monosodium phosphate used increases or as the ratio of disodium phosphate decreases.

From these experiments alone it might be concluded that the OH ion is significant in this oxidation but results given in the next paragraph do not confirm this idea.

When the ratio of the two phosphates is changed by changing the amount of monosodium phosphate but keeping the disodium phosphate constant in amount different results are obtained.

The results in Table IV show that in the presence of a constant amount of disodium phosphate increasing amounts of monosodium phosphate do not retard the oxidation of glucose. In fact the presence of the monosodium phosphate seems to facilitate the completion of the glucose oxidation in spite of the fact that relatively No. 3 is comparable with No. 4 in Table III as far as the proportion of the two phosphates is concerned. Exactly the same result was obtained with Nos. 1 and 2 when they were allowed to react only 2 days. No velocity experiments have been made to determine whether the excess NaH_2PO_4 retards the oxidation as it does the evolution of O_2 from H_2O_2 but the results as given indicate that it does not.

It seems clear that if Löb had done these experiments, as well as some others described below, he would have found it impossible to ascribe so much influence to the OH ions in this catalysis, as he did.

6. Influence of Time on the Oxidation.—In order to follow the glucose oxidation from day to day a large experiment containing

TABLE V.

Glucose.

		gm.
	At beginning contained	0.200
(*a*)	After 24 hrs. "	0.1869
(*b*)	" 48 " "	0.1611
(*c*)	" 72 " "	0.1551*
(*d*)	" 96 " "	0.1212
(*e*)	" 120 " "	0.1173*
(*f*)	" 168 " "	0.0823

* When the results described in this table are plotted the two values marked with the asterisk lie considerably outside the curve. This is due to the fact that undecomposed hydrogen peroxide was still present when the potassium permanganate was added. Thus when two solutions containing exactly the same amount of glucose but one of which also contained 5 cc. of 3 per cent hydrogen peroxide were analyzed, without decomposing the peroxide, the former was found to contain 0.1802 gm. of glucose by the complete oxidation method. The other containing the peroxide apparently contained 0.2165 gm. of glucose when calculated on the basis of the oxygen consumed. This difference is due to the well known fact that hydrogen peroxide reduces permanganate with the evolution of oxygen.

The results are given in this form in order to illustrate this error.

six times as much material as No. 2 in Table II was set up and placed in the incubator at 37°. 75 cc. of this solution (corresponding to 0.200 gm. of glucose) were taken out for analysis at definite intervals as indicated in Table V.

Experiments with the Carbonates of Sodium.

The preceding results clearly confirm Löb's claim that in the presence of phosphate mixture glucose is oxidized by hydrogen peroxide. Since he failed to observe appreciable amounts of oxidation when he used the other common reaction regulator mixtures it seemed unnecessary to test these again for the present. It did seem advisable, however, to make some experiments with the carbonates of sodium for several obvious reasons.

7. Influence of Sodium Carbonate-Bicarbonate.—If the phosphates do not exercise a catalytic effect in this oxidation and the effect observed is due to OH ions then an equimolecular amount of sodium carbonate and bicarbonate should have fully as much effect. That this is not true was definitely established by the following experiment in which 2.43 gm. of $Na_2CO_3.10H_2O$, 0.72 gm. of $NaHCO_3$, 35 cc. of distilled water, 20 cc. (0.200 gm.) of glucose solution, and 20 cc. of 3 per cent H_2O_2 were kept 4 days at 37°. Upon analysis the peroxide was found to have been completely decomposed and equivalent of 0.1902 gm. of glucose was recovered; *i.e.*, 5 per cent was apparently oxidized as against 80 per cent oxidized with the corresponding phosphate mixture.

In the above experiment the two carbonates were used in the same molecular amounts and proportion as the two phosphates in Experiments 5 in Tables I and II. The solution therefore contained at least the same amount of available alkali but had a somewhat higher OH ion concentration than the phosphate mixture referred to. If OH ion concentration and available alkali are the controlling factors in these oxidations this experiment should have shown as much or more oxidation than was obtained with the phosphate mixture.

8. Influence of H_2CO_3 and $NaHCO_3$.—The results in Table V suggest that the velocity of decomposition of sodium bicarbonate, possibly produced in the oxidation, may be a factor in determining the velocity of oxidation. The following three experiments were

done in order to test the influence of this condition. The experiments were set up in similar 250 cc. flasks and kept in the incubator for 24 hours at 37° after which they were analyzed in the usual manner.

(1) 32.0 cc. of 0.33 M NaH_2PO_4 solution + 1.22 gm. of $Na_2CO_3.10H_2O$. This mixture effervesced in the cold. It was heated to boiling to expel CO_2, cooled, and the following were added:

20 cc. (0.200 gm.) of glucose solution, 20 cc. of 3 per cent H_2O_2, 3 cc. of distilled water. 0.0761 gm. of glucose was recovered.

(2) Components the same as in (1).

All ingredients were mixed except the peroxide before adding the $Na_2CO_3.10H_2O$ in order to prevent the loss of CO_2 as much as possible. 0.1906 gm. of glucose was recovered.

(3) 25.6 cc. of 0.33 M Na_2HPO_4 solution.
6.4 " " 0.33 " NaH_2PO_4 "
20.0 " (0.200 gm.) of glucose solution.
20.0 " of 3 per cent H_2O_2.
3.0 " " distilled water.

0.0774 gm. of glucose was recovered.

On the basis of the conditions of the experiments the results of (1) and (3) were expected to be identical because the reaction mixtures as used were identical. As a matter of fact the amount of glucose recovered was nearly the same in (1) and (3). It was expected that the oxidation in (2) would be somewhat slower. In fact only 5 per cent of the glucose was oxidized in (2) as compared with over 60 per cent in the others. This indicates that not only do H_2CO_3 and $NaHCO_3$ not catalyze the oxidation of glucose with hydrogen peroxide but that they actually retard it.

The interpretation of the influence of the sodium carbonate added in (2) has not been fully established as yet. There are several factors to be considered, three of which are as follows: (*a*) Na_2CO_3 may under the conditions in (2) not react completely to give only Na_2HPO_4 and H_2CO_3; (*b*) if so, any Na_2CO_3 or $NaHCO_3$ remaining would rapidly catalytically decompose the H_2O_2; (*c*) the presence of CO_2 to the point of supersaturation may retard the activation or dissociation of H_2O_2.

9. Influence of Sodium Carbonate.—The following experiments were done in order to determine what influence sodium carbonate exercises on the action of disodium phosphate.

(1) 32.0 cc. of 0.33 M Na_2HPO_4 solution.
3.0 " " water.
20.0 " " glucose solution (0.200 gm.).
20.0 " " 3 per cent hydrogen peroxide.
0.0311 gm. of glucose was recovered.

(2) Same as in (1) with 0.61 gm. of $Na_2CO_3.10H_2O$.
0.1741 gm. of glucose was recovered.

(3) Same as (1) with 1.22 gm. of $Na_2CO_3.10H_2O$.
0.1737 gm. of glucose was recovered.

The solutions were kept in the incubator at 37°C. for 45 hours and on analysis the amounts of unchanged glucose given were found. All hydrogen peroxide present had been decomposed.

The results show that sodium carbonate exercises a strongly negative influence on this oxidation in spite of the fact that the OH ion concentration is higher in (2) and (3) and the available alkali in (3) is twice what it was in Experiments 5, Tables I and II.

This negative influence on the final result of the oxidation may be due simply to the fact that the velocity of decomposition of peroxide by Na_2CO_3 is many times greater than that by Na_2HPO_4 and that the glucose oxidation induced by Na_2CO_3 itself is relatively small in comparison.

The above observations on the influence of the carbonates of sodium permit us to conclude that, whatever the mechanism of CO_2 formation in this oxidation may be, carbonates of sodium are not intermediate stages in the process of CO_2 liberation.

10. Influence of Sodium Hydroxide.—The following three experiments were done in order to determine the influence of sodium hydroxide on the effect of the phosphate mixture.

(1) 25.6 cc. of 0.33 M Na_2HPO_4 solution + 6.4 cc. of 0.33 M NaH_2PO_4 + 20 cc. (0.200 gm.) of glucose solution + 20 cc. of 3 per cent H_2O_2 + 3 cc. of water.

(2) The same as (1) except that one-half the water was replaced with 1.5 cc. (0.0857 gm.) of NaOH solution.

(3) The same as (1) except that all the water was replaced with 3 cc. (0.1714 gm.) of NaOH solution.

After 50½ hours at 37°C. the solutions contained no unchanged peroxide. They were analyzed and found to reduce $KMnO_4$ corresponding to glucose as follows:

(1) 0.0088 gm. of glucose.
(2) 0.0140 " " "
(3) 0.0618 " " "

The sodium hydroxide has a perceptible but not a large retarding effect which is interpreted tentatively in the light of other experiments as due simply to its effect in increasing the decomposition of the hydrogen peroxide. In this respect its activity is not so great as that of the carbonates which coincides with its smaller retarding effect on the action of the phosphate mixture.

11. Partial Interpretation of the Influence of Disodium Phosphate.—Since there are three compounds actively concerned in this oxidation reaction and since glucose and peroxide alone do not react appreciably there remain three possible ways of interpreting the reaction on the basis of the formation of molecular complexes, which are so frequently found to underlie catalytic phenomena.

(1) Na_2HPO_4 and H_2O_2 may give an unstable complex which in turn reacts to oxidize glucose.

(2) Na_2HPO_4 and glucose may form a hexose phosphate which is more sensitive to H_2O_2 than free glucose.

(3) The three compounds may form a single complex the instability of which gives rise to the oxidation.

(1) and (2) are readily capable of being tested experimentally by known methods. (3) could conceivably take place in several ways none of which appears to be readily capable of experimental confirmation.

The Na_2HPO_4-H_2O_2 Complex.—That such a complex may be formed is suggested by the experiments of Petrenko on H_2O_2 derivatives of Na_3PO_4.[11] A perphosphate of Na_2HPO_4 is unknown. Moreover perphosphoric acid is apparently unknown.[12] However, pyrophosphoric acid gives a peracid with H_2O_2, stronger

[11] Petrenko, G., *J. russ. phys.-chem. Ges.*, 1902, xxxiv, 204, 391; *Chem. Zentr.*, 1902, i, 1263; ii, 95. *Cf.* also, Gemlin-Kraut, Handbuch der anorganische Chemie, Heidelberg, 7th edition, 1906, i, pt. 1, 146.

[12] Price, T. S., Per-acids and their salts, New York, 1912, 77.

than Caro's acid and which oxidizes Mn to $KMnO_4$ and its sodium salt $Na_4P_2O_7$ gives a stable persalt with 3 per cent H_2O_2.[13]

There is therefore some basis in fact, even in this little studied field, for the idea that Na_2HPO_4 may form an unstable perphosphate as is suggested in the succeeding paragraphs.

Decomposition of Hydrogen Peroxide by the Phosphate Mixture.—Various experiments were done on the influence of Na_2HPO_4 on hydrogen peroxide although it is definitely stated[14] that it is without influence on peroxide. My own experiments, which will not be described here, show that it does decompose hydrogen peroxide and that the presence of an equimolecular amount of NaH_2PO_4 retards but does not stop the decomposition.

TABLE VI.

0.1 N $KMnO_4$ Consumed by 5 Cc. of the Mixture.

No.	23.5 hrs.	47.2 hrs.	96.2 hrs.
	cc.	cc.	cc.
1	22.70	22.62	22.45
2	22.10	21.06	18.45
3	19.35	14.53	2.75
4	17.06	11.65	4.31
5	15.72	9.86	4.08

The only experiments to be described here represent a repetition of the glucose oxidations at 37° in Table II in which the glucose was omitted and in which the peroxide content was determined at intervals during 96 hours. The results given in Table VI indicate the amount of peroxide remaining, at the various intervals, in terms of cc. of 0.1 N $KMnO_4$ consumed by 5 cc. of the mixture.

The phosphate mixtures and the hydrogen peroxide were warmed for 24 hours at 37°C. before being mixed in order to prevent a lag which is otherwise observed during the first 24 hours.

[13] Schenck, R., Vorländer, F., and Dux, W., *Z. angew. Chem.*, 1914, xxvii, pt. 1, 291.

[14] Gemelin-Kraut,[11] p. 137.

The data show a progressively increasing decomposing effect with increasing phosphate content although the mixture is neither appreciably acid nor alkaline.[15]

In conclusion it may be stated that there are clear indications that Na_2HPO_4-H_2O_2 may form an unstable complex, but as yet there is no satisfactory evidence.

The Na_2HPO_4-Glucose Complex.—Harden and Young, von Lebedew, and others[10,16] showed that, in the yeast fermentation of glucose, Na_2HPO_4 combines with glucose to form a hexose phosphate ester. The presence of this complex was demonstrated in part by the fact that much of the phosphate was no longer precipitated with "magnesia mixture." Other hexose phosphoric esters have been obtained by chemical methods[17] but the laboratory preparation of von Lebedew,[16] and the commercial manufacture[18] of hexose phosphate ester are carried out only in the presence of growing yeast. It therefore seemed necessary to determine experimentally whether such a complex is formed in aqueous solutions of glucose and the phosphate mixture alone.

A solution corresponding to No. 5 in Table II except that it contained 20 cc. of water instead of the peroxide solution was kept 3 weeks in the incubator at 37°C. At the end of this time the glucose content was found by reduction methods to be unchanged. After standing 3 weeks more in the laboratory the

[15] It should be noted that the interpretation of this decomposition of H_2O_2 in this case cannot be attributed to the OH ion, since the solution has about the OH ion concentration of water, which is without influence. It is interesting to note in this connection that Schenck, Vorländer, and Dux[13] found that $Na_4P_2O_7$ solutions, which are so alkaline as to feel "soapy," actually stabilize H_2O_2 by forming a stable perpyrophosphate. Moreover, it was found in experiments which will not be given here that the presence of $Na_4P_2O_7$ with Na_2HPO_4 retards or prevents the oxidation of glucose with H_2O_2, but not by decomposing the H_2O_2 as with $NaHCO_3$ or Na_2CO_3.

[16] von Lebedew, A. V., *Biochem. Z.*, 1910, xxviii, 213; 1911, xxxvi, 248. Embden, G., and Laquer, F., *Z. physiol. Chem.*, 1914–15, xciii, 94. Embden, G., Griesbach, W., and Laquer, F., *Z. physiol. Chem.*, 1914–15, xciii, 124.

[17] *Cf.* foot-note, Meyer, V., and Jacobson, P., Lehrbuch der organischen Chemie, Leipsic, 2nd edition, 1902, ii, pt. 2, 927.

[18] *Cf.* for instance Bayer and Company, German Patent 292,817, February 26, 1915; *Chem. Abstr.*, 1917, xi, 1519.

phosphate was precipitated with "magnesia mixture" and weighed as the pyrophosphate. A solution of the phosphates alone made up to the same volume was similarly precipitated at the same time. The two precipitates after ignition showed the same weight, within a small fraction of 1 per cent, which shows that hexose phosphate ester was not formed to any significant extent.

Similar solutions containing glucose and the phosphate mixture were kept under observation in the polariscope in comparison with glucose solutions without phosphates. In 4 days there was no measurable change in optical rotation in either solution.

These data, taken with the absence of positive data in the literature, seem to prove that a hexose phosphate ester such as was found by Harden and Young is not formed under these conditions and consequently has no part in bringing about this oxidation of glucose. If some other type of complex is formed its presence was not demonstrated by these methods.

Influence of Time on the Glucose-Phosphate-Peroxide Reaction.—When it was found that the rate of peroxide decomposition has a definite relation to the phosphate concentration it was of interest to learn what relation the rate of glucose oxidation bears to the rate of peroxide decomposition. The results given in Table VII are typical for the rate of glucose oxidation as obtained by compiling experimental results obtained in conditions like those used for Table II.

In order to test this more fully experiments like Nos. 1 to 5 in Table II were set up having a total volume of 150 cc. and which were placed in the incubator at 37°. The phosphate-glucose mixture and the peroxide were warmed separately for 24 hours before mixing to eliminate what appeared to be a temperature lag in the curves. 10 cc. were removed and analyzed at definite intervals and the results were calculated and recorded in Table VIII on the basis of 75 cc. and thus correspond to the results in Table VII. The materials used in Table VII were not warmed before mixing which accounts for the difference in the slope of the curves when the data are plotted.

The results in both series are substantially the same and show that the rate of glucose disappearance in the presence of the phosphate mixture is appreciably faster than the rate of H_2O_2 disappearance in the absence of glucose (Table VI).

The above constitutes a partial experimental interpretation of the catalytic influence of disodium phosphate on the oxidation of glucose with hydrogen peroxide. The results clearly suggest that the glucose is really oxidized by an unstable disodium perphosphate, formed by the action of peroxide on disodium phosphate.

TABLE VII.

Glucose Remaining from 0.200 Gm. Used.

No.	50 hrs.	97 hrs.
	gm.	*gm.*
1		0.1897
2		0.1843
3		0.1249
4	0.1037	0.0135
5	0.0088	

TABLE VIII.

Glucose Remaining from 0.1902 Gm. Used.

No.	26.5 hrs.	49.2 hrs.
	gm.	*gm.*
1	0.1702	0.1455
2	0.1362	0.1074*
3	0.0489	
4	0.0195	
5	0.0273	

* The behavior of No. 2 is quite variable. Sometimes oxidation is as slow as in No. 1 without phosphates and sometimes it is nearly as fast as in No. 3, but more frequently it is about as given in these results.

12. Is a Glucose-Phosphate Solution Oxidized by Air?—Having shown that the disodium phosphate plays a specific rôle in this catalysis the question arises as to whether the use of peroxide is necessary. A few experiments were done in order to determine whether air could be used instead of peroxide. It is well known that caustic alkalies catalyze the oxidation of sugars by air, with the formation of more or less CO_2 depending on the conditions of the experiment. In the absence of definite data it was possible that the phosphate mixture might play the rôle of caustic

alkali. A mixture like No. 5 in Table II was placed in a wash bottle. A rapid air stream was bubbled through it for 48 hours during 6 days. The permanganate required by 10 cc. was determined at the beginning and at the end of the experiment and showed that no perceptible oxidation had taken place. This shows that disodium phosphate does not act like alkali in this respect, but rather conforms to the rôle of a true peroxidase.[19]

SUMMARY.

1. The work of Löb on the accelerative effect of phosphate mixtures on the oxidation of glucose with hydrogen peroxide was repeated and confirmed.

2. The confirmation consisted in proving by an adequate method that the destruction of glucose, conceded by all in this case, is oxidation.

3. It was shown that glucose may be quantitatively oxidized to CO_2 with hydrogen peroxide in the presence of the phosphate mixture. This fact it appears was not suspected by Löb, and increases the importance of his observations considerably.

4. The results as a whole show that although optimal OH ion concentration is possibly necessary it is less important than

[19] *Cf.* Bach, A., in Oppenheimer, C., Handbuch der Biochemie des Menschen und der Tiere, Jena, 1st edition, 1913, suppl., 160.

Inorganic compounds known to play the rôle of peroxidase in *in vitro* oxidations have usually been, as Bach states, metallic salts of the heavy metals such as iron and manganese. The synthetic peroxidases of Trillat (Trillat, M. A., *Compt. rend. Acad.*, 1904, cxxxviii, 274), of Dony-Hénault (Dony-Hénault, O., *Bull. acad. roy. belg.*, 1908, 105), etc., prepared from manganese were prepared to resemble and imitate what it was thought are the essential properties of an oxidizing enzyme. The peroxidase disodium phosphate differs from these inorganic peroxidases in that the peroxidase property is dependent on the phosphate part of the molecule. Other sodium compounds do not exhibit the same effect. On the other hand dipotassium phosphate, as was shown by experiments not yet published, has the same effect. That the remaining alkali and alkaline earth dibasic phosphates may act in the same way seems likely.

From this point of view then these results are of considerable interest because we have a compound playing the rôle of peroxidase, in which the non-metallic part of the molecule carries the characteristic property. In this respect it seems likely that it resembles the biological peroxidases more closely than the heavy metal derivative peroxidases do.

Löb's experiments and interpretation would indicate, when the phosphate mixture is used, and that the optimal limits, if they exist, are wider than he states.

In fact it seems more accurate to refrain from emphasizing the segregated OH ion in interpreting the reaction and simply state that the effect is specifically related to the presence of disodium phosphate under suitable conditions.

5. The amount of disodium phosphate used is the most significant factor in determining the reaction. Little or much monosodium phosphate was used with a constant amount of disodium phosphate without producing a marked negative effect on the reaction.

6. The phosphate mixture may be used repeatedly at 37° for the oxidation of additional amounts of glucose owing to the fact that the product (CO_2) is evolved from the reaction mixture during the process of oxidation. Disodium phosphate accordingly plays the rôle of a typical catalyst.

7. Consequently disodium phosphate functioning in the manner described in this paper is the only chemical substance known to be generally necessary to the life of organisms, that is known to catalyze the quantitative oxidation of glucose to carbon dioxide.

8. That compounds like hexose phosphate ester are the intermediates involved in the acceleration of oxidation described in this paper seems almost certain at first, in the light of the results of Harden and Young,[10] of von Lebedew,[16] and of Embden, Griesbach, and Laquer.[16] The attempts so far made to establish the formation of such a compound failed to demonstrate its formation under these conditions.

9. On the other hand a close parallelism between the rate of spontaneous decomposition of peroxide and the rate of glucose oxidation in the same solutions was established. This together with other facts developed gives experimental basis for the idea that the oxidation really depends upon the intermediate formation of a highly reactive perphosphate.

10. In producing this accelerating effect upon glucose oxidation disodium phosphate does not play the rôle of both oxygenase and peroxidase, as some inorganic compounds do, but acts only as a peroxidase. It is unable to activate atmospheric oxygen to any appreciable extent.

11. That the phosphate catalysis does not depend alone on unlimited capacity to decompose peroxide is clearly shown by the fact that the hydroxide and carbonates of sodium, which are much more effective in decomposing peroxide, diminish the glucose oxidation roughly in proportion to their increased ability to decompose peroxide.

12. Glucose is not oxidized by hydrogen peroxide in solutions containing $NaHCO_3$ and Na_2CO_3 when these are used in the same molecular concentrations as the two phosphates in the phosphate mixture.

13. These results together with those with sodium hydroxide show that available alkali, contrary to what was observed in the permanganate oxidation of glucose,[4, 20] is without appreciable influence on the oxidation of glucose with hydrogen peroxide.

[20] Witzemann, E. J., *J. Am. Chem. Soc.*, 1917, xxxix, 2657.

STUDIES ON MOLLUSCAN CELOMIC FLUID.

EFFECT OF CHANGE IN ENVIRONMENT ON THE CARBON DIOXIDE CONTENT OF THE CELOMIC FLUID.

ANAEROBIC RESPIRATION IN MYA ARENARIA.

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INTRODUCTION.

It was noted in a previous communication (1) that the content of combined carbon dioxide of molluscan celomic fluid tends to rise when the animals are removed from their natural environment whereas a fall was noticed in this factor in the case of fish removed from their natural habitat. In order to determine what was the cause of this peculiar effect in the molluscan forms a series of experiments was undertaken, the results of which are herein reported.

EXPERIMENTAL.

Effect of Exposure to Atmospheric Air on the Combined Carbon Dioxide Content of the Celomic Fluid.

The method of securing samples of celomic fluid or "clam juice" from the various specimens was the same as that detailed previously (1). Specimens of seven species of pelecypod Mollusca were exposed to atmospheric air in a closed glass container for varying periods of time. One species of the Amphineura and two species of the Gastropoda were similarly studied. Several non-molluscan forms were also exposed to atmospheric air under similar conditions. These included the calcareous shelled arthropod *Balanus aquilla*, the common brachiopod *Terebretella transversa*, various Crustacea of the decapod type, starfish, sea urchins, and certain varieties of marine fish. The container

TABLE I.

No. used.	Specimen.	Time out of water.	CO_2 content of 100 cc. celomic fluid equilibrated with atmospheric air.	Remarks.
		hrs.	*cc.*	
1	*Macoma secta*	0	11.2 } one experiment.	
1	" "	6	48.6 } one experiment.	
1	*Mya arenaria*	0	11.2 } one experiment.	
1	" "	17	27.3 } one experiment.	
1	" "	1	19.9	
1	" "	4	19.5	
1	" "	0	10.2 } one experiment.	
1	" "	24	32.3 } one experiment.	Same specimen as above.
1	" "	24	28.2	
9	" "	0	8.0	Kept for some days in floating carriage attached to landing stage.
11	" "	28	36.0	Kept for some days in floating carriage attached to landing stage.
3	" "	25	34.8	
3	" "	24	28.2	
4	" "	24	38.0	Kept in sealed container placed in sea water off landing stage.
4	" "	120	55.0	
12	" "	90	44.0	
12	" "	0	6.5 } one experiment.	Kept for some days in floating carriage attached to landing stage.
10	" "	48	65.0 } one experiment.	Kept in sealed container placed in sea water off landing stage.
11	" "	96	74.5 } one experiment.	Kept in sealed container placed in sea water off landing stage.

6	*Mya arenaria*.....	0	6.5	one experiment.	Kept for some days in floating carriage attached to landing stage.
4	" "	24	38.0		Kept in sealed container placed in sea water off landing stage.
10	" "	48	69.0		Kept in sealed container placed in sea water off landing stage.
8	" "	72	78.2		Kept in sealed container placed in sea water off landing stage.
2	" "	96	105.0		Kept in sealed container placed in sea water off landing stage.
11	*Paphia staminea*.....	0	12.8		
1	" "	0	27.9		
3	" "	6	39.0		
1	" "	6	34.5		
1	" "	16	40.0		
1	" "	44	59.0		
8	" "	28	39.5		
1	*Schizothoerus nuttalli*.....	0	8.3		
1	" "	12	23.2		
3	" "	32	30.7		
1	*Saxidomus gigantea*.....	0	8.1		
1	" "	12	24.2		
1	" "	6	29.0		
1	" "	24	40.0		
8	" "	30	44.0		
1	" "	30	53.0		

TABLE 1.—*Concluded.*

No. used.	Specimen.	Time out of water.	CO_2 content of 100 cc. celomic fluid equilibrated with atmospheric air.	Remarks.
		hrs.	*cc.*	
1	*Cardium corbis*.....	0	7.9	
5	" "	2	12.6	
1	" "	6	14.9	
1	" "	14	16.5	
1	" "	24	18.6	
8	" "	24	16.3	
5	" "	30	20.0	
4	" "	32	18.0	
1	*Mytilus edulis*.....	0	8.1	
1	" "	0	9.9	
1	" "	30	27.3	
1	*Cryptochiton*.....	0	9.0	
1	"	16	18.0	
1	*Polynices* (Lunatia) *lewisii*.....	0	12.5	
1	" " "	24	31.0	
1	" " "	30	77.4	
1	" " "	52	61.2	
1	" " "	52	63.0	
1	*Thais lamellosa*.....	0	18.5	
1	" "	10	28.2	
1	" "	24	45.8	
1	*Balanus aquilla*.....	0	11.2	
1	" "	9	17.7	Same specimen as above.

1	*Terebretella transversa*.....	0	6.8	
3	" "	30	12.7	
1	*Strongylocentrotus drobachiensis.*	0	10.2	
1	" "	4	14.8	Same specimen as above.
1	" "	0	10.2	
1	" "	8	6.6	Same specimen as above.
1	*Pisaster ochracea*.....	0	6.5	
1	" "	8	6.5	Same specimen as above.
1	" "	32	7.0	
1	" "	0	7.0	
1	" "	24	10.0	Same specimen as above.
1	" "	0	6.0	
1	" "	24	7.0	Same specimen as above.
1	*Cancer magister*.....	0	18.0	
1	" "	5	18.0	Same specimen as above.
1	" "	0	18.4	
1	" "	8	19.5	Same specimen as above.
1	*Platichtys*, plasma.....	0	10.2	
1	" whole blood.....	0	3.7	Dead some hrs.
1	*Sebastodes*.....	0	11.2	
3	"	3	7.3	
1	"	0	9.3	On line ½ hr.
1	*Squalus sucklii*.....	0	5.6	
1	" "	4	3.7	

used was of good size and a full supply of oxygen was assured. It was kept covered to prevent loss of water by evaporation. Filter paper moistened in sea water was frequently placed in the container with the specimens. Specimens which were exposed to air were kept in certain instances at a fairly constant temperature by immersing the container in sea water while in others they were kept in the laboratory and were thus subject to the temperature changes of the latter. Table I illustrates the effects of exposure to air for various periods upon the combined carbon dioxide content of the celomic fluid of the different species investigated. The rapid increase in the carbon dioxide content of the celomic fluid of the molluscan forms and the arthropod *Balanus aquilla* is very striking. That this increase is due to bicarbonate is evident since the samples were equilibrated with atmospheric air before being submitted to analysis. The decapod crustaceans examined failed to show this reaction, while a very slight increase was in some instances manifested in the echinoderms. As the latter reaction was not uniform it is of very doubtful significance.

The survival time for specimens exposed to atmospheric air varied greatly. It was early noted that *Mya arenaria* was peculiarly resistant to long exposure to atmospheric air at the temperatures which prevailed in the surface water and the air at Departure Bay during the summer months. It was for this reason used extensively in later investigation. It is regretted that no facilities were available which would enable one to keep specimens at a low as well as constant temperature. The results obtained will therefore have to be considered in the light of this condition. The greatest increase in the carbon dioxide content of the blood was in two specimens of *Mya arenaria* which had been exposed for 96 hours. The increase here was from 6.5 volumes per cent in the controls kept in sea water to 105 volumes per cent in the specimens exposed to atmospheric air at the temperature of surface sea water. The container used was a 6 liter cylindrical glass museum jar and it was opened daily both for the purpose of removing specimens for examination and to allow a change of air. The temperature of the sea water in the vessel which was taken on the 4 consecutive days during which these specimens were exposed was 19.8°, 18.8°, 19.3°, and 18.9°C. Slightly over a

sixteenfold increase in the combined carbon dioxide in the blood was noticed in this instance.

The increase in the carbon dioxide in the small pelecypod *Macoma secta* from 11.2 to 48.6 volumes per cent in 6 hours is noteworthy, as is also that observed in the gastropod *Polynices lewisii* following 30 hours exposure. The combined carbon dioxide rose in this latter instance from 12.5 volumes per cent in the control to 77.4 volumes per cent in the exposed specimen. There were few forms which would survive an exposure to atmospheric air of more than 24 hours at the prevailing land and surface water temperatures. The amphineuran *Cryptochiton*, the cockle *Cardium corbis*, and the eagle barnacle *Balanus aquilla* were very sensitive to exposure. The horse clam *Schizothoerus nuttalli*, and the butter clam *Saxidomus gigantea* withstood an exposure of 24 to 48 hours. The little neck clam *Paphia staminea* and the edible form *Mya arenaria* were very resistant to exposure and in some instances survived as long as 5 days when placed in the air, the evaporation of water being practically excluded. The nudibranch *Anisodoris* was most sensitive of all forms, dying shortly after being brought into the laboratory. The absence of a calcareous shell is probably associated with the lack of resistance to exposure to air in this form although the temperature factor must also be of great importance.

Effect of Exposure to Atmospheric Air on the Carbon Dioxide Capacity of the Celomic Fluid.

Several samples of celomic fluid taken from specimens exposed to atmospheric air for varying periods were analyzed in the Van Slyke apparatus (2) when equilibrated with atmospheric air and also when equilibrated with alveolar air of the normal subject after the manner described by Van Slyke and Cullen (3). Table II illustrates the results obtained in this series of experiments. It will be noted that in every instance the carbon dioxide capacity of the sample was considerably in excess of the carbon dioxide content of the same when equilibrated with atmospheric air.

TABLE II.

No. used.	Specimen.	CO_2 content of 100 cc. celomic fluid.		Remarks.
		Equilibrated with atmospheric air.	Equilibrated with alveolar air.	
		cc.	cc.	
1	*Echidnocerus formatus*	22.5	28.0	3 hrs. out of water.
1	*Pisaster ochracea*	4.1	9.8	Fresh.
1	" "	7.4	11.2	9 hrs. out of water.
1	" "	7.0	12.2	32 " " " "
1	*Strongylocentrotus drobachiensis*	7.4	9.8	Fresh.
1	*Dermasterias imbricata*	5.5	8.8	"
1	*Schizothoerus nuttalli*	9.9	14.4	"
3	" "	30.7	39.8	30 hrs. out of water.
1	*Balanus aquilla*	11.2	18.6	Fresh.
3	*Mya arenaria*	34.8	39.4	25 hrs. out of water.
10	" "	42.0	50.0	60 " " " "
4	" "	17.6	23.3	24 " in boiled sea water in sealed container.
11	" "	36.0	40.0	28 hrs. out of water.
3	" "	28.2	33.0	24 " " " "
10	" "	6.5	13.0	Fresh.
2	" "	18.8	25.4	25 hrs. in desiccator over alkaline pyrogallol.
8	*Paphia staminea*	39.5	45.0	28 hrs. out of water.
1	*Cardium corbis*	12.6	18.0	2 " " " "
1	*Saxidomus*	39.6	43.2	30 " " " "
1	*Polynices lewisii*	77.4	82.8	30 " " " "
1	" " (fluid from foot)	16.5	21.5	Fresh.

Effect of Exposure to Atmospheric Air on the Total Nitrogen Content of the Celomic Fluid.

In order to determine if the increase in the carbon dioxide content of molluscan celomic fluid brought about by exposure to atmospheric air was in any way due to an increase in its protein content the total nitrogen was determined in from 25 to 100 cc. of composite samples of celomic fluid taken from several specimens. The estimation was made by the usual Kjeldahl method. The results are expressed in Table III. As there was a slight

TABLE III.

No. used.	Specimen.	Total nitrogen per 100 cc. of fresh material.	Total nitrogen per 100 cc. after exposure to atmospheric air.	Time in air.
		mg.	*mg.*	*hrs.*
1	*Schizothoerus nuttalli*	37.5		0
2	" "	34.0		0
3	" "		34.4	32
15	*Saxidomus gigantea*	50.3		0
8	" "		51.8	30
23	*Paphia staminea*	70.0		0
8	" "		61.6	28
6	*Cardium corbis*	37.5		0
6	" "		50.4	30
14	*Mya arenaria*	33.6		0
11	" "		40.7	28
1	*Polynices lewisii* (fluid from foot)		9.6	52

increase in two species, *Cardium corbis* and *Mya arenaria*, practically no change in *Saxidomus gigantea*, and a slight decrease in *Schizothoerus nuttalli* and *Paphia staminea*, it is very unlikely that protein plays any appreciable part in the increase in the combined carbon dioxide or alkali reserve of the celomic fluid of the mollusk which has been exposed to atmospheric air.

Effect of Exposure to Atmospheric Air upon the Calcium and Magnesium Content of the Celomic Fluid.

Calcium and in a few instances magnesium were determined in composite samples of celomic fluid taken from several specimens which had been exposed to atmospheric air for varying

TABLE IV.

No. used.	Specimen.	CO_2 per 100 cc. of fluid equilibrated with atmospheric air.	Ca per 100 cc.	Mg per 100 cc.	Alkalinity.	Reactivity.	Remarks.
		cc.	*mg.*	*mg.*	*cc. 0.01 N NaOH*	*cc. 0.01 N H_2SO_4*	
	Experiment I.						
10	*Mya arenaria*.....	6.5	35.4	49.0	−9.0	90.0	Fresh. Kept some days in floating carriage off landing stage.
4	" "	38.0	92.0	79.0			24 hrs. in air at temperature of sea water.
10	" "	69.0	186.0	82.0	−24.0	720.0	48 " " " " " " " "
8	" "	78.2	220.0		−60.0	1,080.0	72 " " " " " " " "
2	" "	105.0	240.0	90.0	−130.0	1,140.0	96 " " " " " " " "
	Experiment II.						
10	*Mya arenaria*.....	6.5	30.0	44.5			Fresh. Kept some days in floating carriage attached to landing stage.
10	" "	65.0	135.0	48.0			48 hrs. in air at temperature of sea water.
11	" "	74.5	223.0	57.3			96 " " " " " " " "
	Experiment III.						
2	*Mya arenaria*.....	7.5	38.0		−6.0	76.0	Fresh.
3	" "	38.7	140.0		−18.0	450.0	24 hrs. in air at temperature of sea water.
4	" "	61.2	208.0		−30.0	690.0	48 " " " " " " " "
3	" "	72.5	231.0	75.0	−24.0	860.0	72 " " " " " " " "
1	" "	84.0					96 " " " " " " " "
	Experiment IV.						
5	*Cardium corbis*....	9.3	48.0	78.0	−17.6	140.0	Fresh from Sand Beach.
8	" "	16.3	84.0	78.0	−32.0	200.0	Kept 24 hrs. in air in laboratory.
	Experiment V.						
5	*Cardium corbis*....	8.4	48.0	100.8	−16.0	95.0	Fresh from Sand Beach.
7	" "	19.0	98.0	100.2	−30.0	220.0	Kept 20 hrs. in air in laboratory.

periods of time. The method of McCrudden (4) was followed, the calcium being estimated by the titration of the oxalate with 0.1 N potassium permanganate. 25 cc. of celomic fluid were used in most instances. This was evaporated to dryness on the water bath, fused, dissolved by the aid of concentrated hydrochloric acid, and the calcium finally precipitated as the oxalate. The results are shown in Table IV. It will be noted that, whereas magnesium increases only very slightly in the blood of an exposed mollusk, the calcium increases to a great extent and also the increase in this latter constituent is more or less parallel with the increase in the combined carbon dioxide. It is therefore evident that the great increase in the alkali reserve of the blood of molluscan forms when exposed to atmospheric air is due to an increase in the concentration of bicarbonate which is balanced for the most part by an increase in the concentration of the calcium ions. Myers (5) has reported finding 307 mg. of calcium calculated as oxide in the blood of *Saxidomus nuttalli* and 197 mg. in *Schizothoerus nuttalli*. He has also commented on the very high calcium content of molluscan blood. I have failed to find that the calcium content of the celomic fluid of fresh molluscan forms differs materially from that of sea water. It is only after exposure to air that the calcium content becomes high.

Effect of Exposure to Atmospheric Air upon the Total Alkalinity and the Buffer Value or Reactivity of the Celomic Fluid.

Lacking the means of determining the hydrogen ion concentration, a most important factor in these experiments, a method was employed to determine approximately the total alkalinity of the blood and also its buffer value. The method adopted was similar to that previously described (1) based on the principle made use of in the method of double titration for bicarbonates by Brown and Escombe (6). The presence of small amounts of protein would of course introduce an error but as has been shown the protein content of the celomic fluid does not vary to any appreciable extent and therefore approximately the same degree of error would exist in all the titrations. The alkalinity was determined by noting the amount of 0.01 N alkali required to produce a just noticeable pink tint when phenolphthalein had

been added to the celomic fluid. The reactivity of Moore and Wilson (7) or the buffer value of Sörensen (8) was determined by titrating from the phenolphthalein to the methyl orange point using 0.01 N sulfuric acid. 0.2 N acid was used in those instances where the reactivity was of large proportion. The results are shown in Table IV. It will be noted that the rate of increase in the reactivity of the celomic fluid is in close agreement with the rate of increase of calcium and also of the combined carbon dioxide content of the same.

Effect of Exposure to Air Followed by Submersion in Fresh Water.

Table V illustrates that, whereas exposure to air causes a rapid increase in the alkali reserve of the celomic fluid, the subsequent immersion in fresh sea water causes a return to approximately the normal value for this factor.

TABLE V.

Specimen.	CO_2 content of 100 cc. of fluid equilibrated with atmospheric air.	Remarks.
	cc.	
Mya arenaria	8.2	Fresh.
" "	32.0	72 hrs. in glass container in laboratory.
" "	23.3	Submerged 4 hrs. in fresh sea water.
" "	14.9	" 16 " " " " "
" "	9.2	" 64 " " " " "

Effect of Submersion in Sea Water in a Sealed Container.

Several fresh specimens of *Mya arenaria* were immersed in a relatively small volume of sea water in a cylindrical glass container which was then tightly sealed. After varying periods of time the celomic fluid of the specimens was examined. An analysis of the sea water which was used in the experiment was also made. The results are expressed in Table VI. A few experiments were carried out in which boiled out sea water was used in place of fresh sea water. In one instance sea water, the buffer value of which had been greatly increased by the addition of 5 gm. of basic sodium phosphate per liter, was used. It will

be noted that the behavior of *Mya arenaria* kept in a sealed container in either boiled or fresh sea water is very similar to that observed when the specimens were kept in the air. The alkali reserve and the calcium content of the celomic fluid mount steadily until the animal dies. The increase in the carbon dioxide and calcium content of sea water is also considerable. The values for these two factors in sea water are, however, lower in the case of the celomic fluid except after long submersion of the specimens, in which instance there is a tendency for the concentrations of these latter substances in the celomic fluid and sea water to equalize. The addition of basic sodium phosphate to the boiled sea water used in one experiment did not materially alter the results. A dense precipitate was formed in the sea water in this experiment which consisted for the most part of calcium phosphate.

If one considers the increase in the combined carbon dioxide in the sea water used in an experiment one finds that there is very little difference in the rate of increase in this factor in specimens exposed to air and in specimens submerged in a relatively small volume of sea water. Thus in Experiment I, Table VI, an increase of 29.3 volumes per cent was noted in the combined carbon dioxide content of the celomic fluid, while an increase of fully 20 volumes per cent took place in the sea water. The total bulk of the eight specimens used in this experiment was 550 cc. while the volume of sea water used was 750 cc. There was therefore an increase of 150 cc. of combined carbon dioxide due to the activity of the specimens over and above the increase noted in their celomic fluid. The shells of the eight specimens displaced 85 cc. of water. Not allowing for the water entrapped in the mantle cavities, there were 465 cc. of clam tissue present in this experiment so that the increase of 150 cc. of carbon dioxide found in the sea water would mean that 32 cc. of this carbon dioxide had resulted from the activity of each 100 cc. of clam tissue. This added to the carbon dioxide content of 100 cc. of celomic fluid indicates that approximately 61.3 cc. of combined carbon dioxide resulted from the activity for 46 hours of 100 cc. of clam tissue, an amount which is in close agreement with the observed increase of the volume per cent of carbon dioxide in the celomic fluid of similar specimens exposed to atmospheric air in a closed vessel for a corresponding period of time (Table IV).

TABLE VI.

No. used.	Specimen.	CO_2 per 100 cc. equilibrated with atmospheric air.	Ca per 100 cc.	Mg per 100 cc.	P_2O_5 per 100 cc.	Alkalinity.	Reactivity.	Remarks.
		cc.	*mg.*	*mg.*	*mg.*	*cc. 0.01 N NaOH*	*cc. 0.01 N H_2SO_4*	
	Experiment I.							
10	*Mya arenaria*.....	7.5	38.0			−6.0	74.0	Fresh.
8	" " (550 cc.).....	36.8	149.0			−25.0	550.0	46 hrs. in 750 cc. sea water in sealed container at temperature of sea water.
	Sea water (750 cc.).....	23.4	102.0	95.0		−13.5	140.0	8 *Mya arenaria* in this 46 hrs.
	Experiment II.							
	Boiled sea water.....	2.2	33.0			3.0	11.3	
10	*Mya arenaria*.....	6.5	35.4	49.0		−9.0	90.0	Fresh control.
1	" "	31.5						One of fifteen in boiled sea water in sealed container 42 hrs. All alive.
12	" "	51.0	121.0					90 hrs. in boiled sea water; 3 dead, 12 living; analysis of latter only.
	Boiled sea water (700 cc.)....	51.0	121.0			−21.4	350.0	15 *Mya arenaria* (600 cc.) in this 90 hrs.
	Experiment III.							
	Boiled sea water.....	2.2			273.0	−30.0	160.0	5 gm. of Na_2HPO_4 added per liter.

11	*Mya arenaria*.....	6.5	29.0			−11.2	80.0	Fresh control.
11	" "	26.6	125.0		51.0	−70.0	480.0	All alive (600 cc.). 44 hrs. in boiled sea water as above.
	Boiled sea water (700 cc.)....	26.6	50.0	50.0	50.0	−82.0	265.0	11 *Mya arenaria* in this 44 hrs.
	Experiment IV.							
	Boiled sea water.....	3.7						
10	*Mya arenaria*.....	6.5	56.0	66.0		4.0	16.7	
4	" "	17.5				−9.0	90.0	Fresh control.
	Boiled sea water (250 cc.)....	9.4				−5.5	54.0	24 hrs. in boiled sea water (250 cc.). All alive. 4 *Mya arenaria* in this 24 hrs.

TABLE VII.

No. used.	Specimen.	CO_2 per 100 cc. equilibrated with atmospheric air.	Ca per 100 cc.	Mg per 100 cc.	Cl	Alkalinity.	Reactivity.	Remarks.
		cc.	*mg.*	*mg.*	*per cent*	*cc. 0.01 N NaOH*	*cc. 0.01 N H_2SO_4*	
	Experiment I.							
12	*Mya arenaria*..	6.5	35.4	49.0		−9.0	90.0	Fresh control.
20	" " ..	25.0	84.0	28.5		−24.0	330.0	45½ hrs. in distilled water in sealed container. 10 alive, 1 dead. Bulk of clams 390 cc. Water 400 cc.
	400 cc. water...	13.0	30.0	15.0		−11.0	95.0	11 *Mya arenaria* in this 45½ hrs.
	Experiment II.							
6	*Mya arenaria*..	7.5	38.0		1.301	−9.0	90.0	Fresh. Cl in sea water 1.303 mg.
6	" " ..	19.5	77.6	24.0	0.747	−9.0	250.0	24 hrs. in fresh distilled water in sealed container. Bulk of clams 530 cc. Water 770 cc.
	770 cc. water...	4.0	15.0	10.0	0.211	−3.5	38.0	6 *Mya arenaria* in this 24 hrs.
	Experiment III.							
2	*Mya arenaria*..	20.5			0.802			20 hrs. in distilled water in sealed container. Bulk of clams 200 cc. Water 1,100 cc.
	1,100 cc. water.				0.132			2 *Mya arenaria* in this 20 hrs.

Effect of Submersion in Distilled Water.

The results of some experiments in which specimens of *Mya arenaria* were submerged in fresh distilled water in a glass container and kept at the temperature of surface sea water appear in Table VII. The combined carbon dioxide and calcium content of celomic fluid rise after much the same manner as was observed when specimens were placed in either fresh or boiled sea water. Experiments I and II, Table VII, are of interest in that they show that a fall had taken place in the concentration of magnesium in the celomic fluid of the specimens, while a rise occurred in the concentration of calcium. There is also a marked difference in the total alkalinity and in the reactivity of the water and the celomic fluid. The chlorine content of the celomic fluid and of the water in which the specimens of *Mya arenaria* were submerged was determined in Experiments II and III, Table VII. The manner in which the concentration of the chloride in the celomic fluid is kept at a relatively high level under the circumstances obtaining in these experiments is remarkable. Meigs (9) has shown that the adductor muscle of the clam *Venus mercenaria* is peculiarly resistant to hypertonic solutions of sodium chloride and to double strength sea water, the concentration of sodium chloride rising to only about one-half that of the surrounding medium. He also found that the mantle is almost impermeable to sodium chloride. The ability of *Mya arenaria* to withstand immersion in water, the osmotic pressure of which is very low, is of interest in the light of the observation of Meigs.

Effect of Exposure to a Hydrogen Atmosphere.

Several specimens of *Mya arenaria* were kept in a small volume of sea water for 2 hours in order that the oxygen content of their tissues should be reduced to a low level. One specimen was then bled as a control, the others were placed in a hydrogen atmosphere over alkaline pyrogallic acid, two separate containers being used. One group of three was exposed for 26 hours; the other for 48 hours. The results of the analyses of the celomic fluids of these specimens are shown in Table VIII. The maximum and minimum temperatures for the 2 days during which

TABLE VIII.

No. used.	Specimen.	CO_2 per 100 cc. of fluid equilibrated with atmospheric air.	Ca per 100 cc.	Mg per 100 cc.	Alkalinity.	Reactivity.	Remarks.
		cc.	*mg.*	*mg.*	*cc. 0.01 N NaOH*	*cc. 0.01 N H_2SO_4*	
1	*Mya arenaria*....	8.2					Kept in small volume of sea water 2 hrs.
3	" "	26.4	156.0	75.0	−16.0	410.0	Kept in hydrogen atmosphere over alkaline pyrogallol 26 hrs.
3	" "	45.5			−22.0	690.0	Kept in hydrogen atmosphere over alkaline pyrogallol 48 hrs.
4	" "	53.0			−45.0	950.0	Kept in hydrogen atmosphere 56 hrs.

this experiment was performed were on the 1st day 24.4° and 10.9°C., on the 2nd day 23.9° and 11.4°C. It will be noticed that the increase observed in the concentration of bicarbonate in the celomic fluid is very marked. It is not, however, so great as that which is found when the specimens are exposed to atmospheric air. The increase in the calcium and magnesium is comparable to that observed for bicarbonate.

Effect of Exposure to a Nitrogen Atmosphere.

Three specimens of *Mya arenaria* were placed in a small desiccator containing a concentrated solution of pyrogallic acid in 40 per cent sodium hydroxide. A glass tube was so attached

TABLE IX.

No. used.	Specimen.	CO_2 per 100 cc. of fluid equilibrated with atmospheric air.	Remarks.
		cc.	
3	*Mya arenaria*	8.0	Fresh control.
3	" "	34.8	25 hrs. in glass container in laboratory.
3	" "	17.8	25 " " desiccator over alkaline pyrogallol.

to the exhaust cock that as oxygen was absorbed the air which entered first bubbled through the alkaline pyrogallol in the bottom of the desiccator. Three other specimens of like size were placed at the same time in a glass container which was kept at the same temperature as the desiccator for the duration of the experiment. After 25 hours the specimens were bled and an analysis was made of the composite samples of celomic fluid. The results are expressed in Table IX. It will be observed that the combined carbon dioxide of the celomic fluid did not increase to the same extent in the specimens which were kept in the desiccator over alkaline pyrogallol as it did in the controls which were kept in the air.

TABLE X.

Specimen.	Fluid examined.	CO_2 per 100 cc. equilibrated with atmospheric air.	Remarks.
		cc.	
Mya arenaria.....	Celomic fluid from sinus.	8.3	Fresh.
" "	Fluid from exhalent siphon.	4.6	" Same specimen as above.
" "	Celomic fluid from sinus.	39.6	25 hrs. out of water.
" "	Fluid from mantle cavity.	36.0	25 " " " " Same specimen as above.
Cryptochiton.....	Celomic fluid from sinus.	9.0	Fresh.
"	Fluid from foot.	3.6	" Same specimen as above.
Polynices (Lunatia) *lewisii*.	Celomic fluid from sinus.	20.7	Fresh.
" " "	Fluid from foot.	17.1	" Same specimen as above.
" " "	Celomic fluid from sinus.	21.0	"
" " "	Fluid from foot.	16.5	" Same specimen as above.
" " "	Celomic fluid from sinus.	14.9	"
" " "	Fluid from foot.	12.5	" Same specimen as above.
" " "	Celomic fluid from sinus.	31.0	24 hrs. out of water.
" " "	Fluid from foot.	31.0	24 " " " " Same specimen as above.
" " "	Celomic fluid from sinus.	12.1	Fresh.
" " "	Fluid from foot.	12.1	" Same specimen as above.
" " "	Celomic fluid from sinus.	65.7	52 hrs. out of water.
" " "	Fluid from foot.	63.0	52 " " " " Same specimen as above.
" " "	Celomic fluid from sinus.	77.4	30 " " " "
" " "	Fluid from foot.	59.4	30 " " " " Same specimen as above.
" " "	Celomic fluid from sinus.	61.2	52 " " " "
" " "	Fluid from foot.	37.8	52 " " " " Same specimen as above.

Comparison of the Carbon Dioxide Content of Celomic Fluid and Other Fluids Obtained from Mollusca.

A comparison was made between the carbon dioxide content of the celomic fluid and other fluids of different Mollusca. The results of this study are shown in Table X. It was found that the carbon dioxide content of the fluid which exuded from the exhalant siphon of fresh *Mya arenaria* was just slightly higher than that of sea water. When specimens of this species were exposed to atmospheric air for some time the bicarbonate content in the fluid of the mantle cavity closely approximated that in the celomic fluid. Somewhat similar observations were made on the amphineuran form *Cryptochiton*. The fluid in the foot of the large gastropod *Polynices lewisii* bears somewhat different relation to the blood and celomic fluid of this form as far as the bicarbonate content is concerned from that which holds in the fluid between the mantle cavity and the blood and celomic fluid of a pelecypod such as *Mya arenaria*. The combined carbon dioxide content of the fluid of the foot of *Polynices lewisii* approximates the value observed for the celomic fluid. The drawing in, therefore, of the foot in this form causes a considerable decrease in the total alkali reserve of the animal. It is of interest in this connection to note that this animal does not withdraw its foot unless subjected to rather violent irritation.

Effect of Submersion of Dead Specimens of Mya arenaria in Sea Water.

The results of two experiments are shown in Table XI. It is evident that the bicarbonate content of sea water in which dead clams are immersed rises quite rapidly once decomposition has set in. It will be noted, however, that there is little change during the first 24 hours submersion. The behavior of pelecypod mollusks exposed to air or submerged in a relatively small volume of water is therefore quite distinct from that of dead clams which are undergoing decomposition.

TABLE XI.

Specimen.	Total CO_2 per 100 cc.	Remarks.
	cc.	
Experiment I.		
4 *Mya arenaria* (175 cc.)		Placed in 275 cc. of boiling sea water.
Sea water (boiled)	3.3	
" " "	4.7	After 24 hrs. No decomposition of clam tissue.
" " "	42.0	After 48 hrs. Decomposition clearly manifested.
" " "	55.0	After 72 hrs.
" " "	67.0	" 96 "
" " "	72.0	" 144 "
Experiment II.		
10 *Mya arenaria* (500 cc.)		Placed in 700 cc. of boiled sea water containing 9.5 per cent alcohol.
Sea water (boiled)	2.8	
" " "	3.3	After 24 hrs.
" " "	11.7	" 48 " Milky, decomposition evident.
" " "	35.0	After 96 hrs.

DISCUSSION.

As has already been indicated the marked increase in the bicarbonate content of the celomic fluid, and therefore in all probability of the blood and tissues of the calcareous shelled pelecypod mollusks and the arthropod *Balanus aquilla* on exposure to air is quite opposite to the effect observed in fishes when they are removed from their natural habitat. This phenomenon is undoubtedly associated with the presence of a calcareous shell the calcium carbonate of which furnishes an alkali reserve which is added to that of the body fluids and tissues, and which it appears can be readily utilized.

As specimens of *Mya arenaria* appear to remain practically normal even after long exposure to atmospheric air there is no reason to suppose that any material change has been effected in their metabolic processes as a result of the change in environment. If one assumes, therefore, that combustion still proceeds in the

exposed specimens, then the increase in the bicarbonate content of the body fluids can be explained according to the equation

$$CO_2 + H_2O + CaCO_3 \rightleftharpoons Ca(HCO_3)_2$$

The carbon dioxide resulting from the respiratory process would, by slightly increasing the hydrogen ion concentration, dissolve calcium carbonate from the shell and the concentration of the calcium ions and of bicarbonate ions would therefore steadily rise as combustion in the tissues proceeded. The amount of carbon dioxide actually excreted from the specimens in the gaseous form was not determined. If this factor were known one could calculate the intensity of metabolism in these forms by considering the amount of carbon dioxide excreted in addition to the amount retained as bicarbonate. It would appear that 50 per cent of the increase observed in the carbon dioxide content of the celomic fluid is due to carbon dioxide formed by combustion in the tissues, while the remaining 50 per cent results from the solution of calcium carbonate of the shell.

The degree of alkalinity of the celomic fluid determined by titration is by no means an indication of the hydrogen ion concentration, but the ratios observed between the alkalinity figures and the reactivity values suggest that no marked increase in the hydrogen ion concentration takes place during the early part of the exposure at least. The reactivity or buffer value is, in nearly every instance, in close agreement with the calcium content and the carbon dioxide concentration of the celomic fluid.

The increase in the alkali reserve as indicated by an increase in bicarbonate concentration in specimens exposed to atmospheric air is due for the most part to increase in the calcium content. Magnesium, which in the normal animal in its natural habitat exceeds calcium in the degree of its concentration, and therefore balances a greater proportion of bicarbonate ions than does calcium, increases only slightly as compared with calcium when a specimen is exposed to air. It is probable that the relative increase in calcium and magnesium concentrations under these circumstances is somewhat similar to the relative amounts of these substances in the shell from which solution of bicarbonate is taking place. It is of interest to note here that no increase

was observed in the concentration of magnesium in the cockle (*Cardium corbis*) on exposure to air.

As specimens submerged in boiled sea water and kept in a sealed container continue to develop an increased carbon dioxide content, calcium concentration, and buffer value, after much the same manner as specimens exposed to atmospheric air, and since a similar effect is manifested by specimens kept in an atmosphere of hydrogen or nitrogen, one is led to ask the question "Can anaerobic respiration be manifested by these forms?"

If one considers the results of an experiment recorded in Table VIII, one finds that after 48 hours in a hydrogen atmosphere the combined carbon dioxide rose from 8.2 to 45.5 volumes per cent, or an observed increase of 37.3 volumes per cent. If one assumes that aerobic respiration was taking place and that carbohydrates were being burned, then a volume of oxygen equivalent to the volume of carbon dioxide produced would be required. If 50 per cent of the observed increase in the combined concentration of carbon dioxide is indicative of the amount of this substance produced due to combustion then an amount of oxygen equivalent to 50 per cent of 37.3 volumes per cent, or 18.65 volumes per cent, would be required. As the specimens used in this experiment were kept in a small volume of sea water for 2 hours before they were transferred to a hydrogen atmosphere, one fails to see how any appreciable amount of oxygen could be contained in the tissues of the specimens. As there is no apparent source for 18.65 volumes per cent of oxygen in these clams it is therefore evident that they must be respiring anaerobically or else the increase in the carbon dioxide, calcium, and buffer value of the celomic fluid is due to some other cause than that suggested earlier in the paper. Decomposition of the clam tissue can be excluded since the specimens were very active, responding to stimulation like normal animals, after they were removed from the hydrogen atmosphere.

There is the possibility of the solution of the calcium carbonate of the shell due simply to the solvent action of the tissue fluids containing free carbon dioxide. This would result in the formation of calcium carbonate the solubility of which is considerably increased by an excess of carbon dioxide in the water (10). In dealing with a closed system, however, such as the individual

clam in an atmosphere of hydrogen, the solution of calcium carbonate due to the solvent action of the free carbon dioxide would require a constant supply of the latter if the process is to continue; otherwise equilibrium would be established between the dissolved bicarbonate, the calcium carbonate of the shell, and the free carbon dioxide, and no further increase would be manifested unless this balance were disturbed. It will be noted that the rate of increase in the carbon dioxide content and the calcium concentration of the blood is for a considerable period practically constant. If respiratory activity accounts for the increase in bicarbonate concentration of the tissue fluids, then this uniformity in the rate observed would fit in well with the fairly constant rate of metabolism which might be expected under such circumstances, anaerobic respiration being possible.

The fact that the rate of increase in the bicarbonate concentration, the calcium content, and the buffer value is greater in air than it is in either hydrogen or nitrogen would indicate that absence of oxygen does exert an influence on the intensity of the metabolic processes but by no means causes a complete cessation in the respiratory function.

The extreme sensitivity of the cockle and the horse clam to exposure to air at the prevailing summer temperatures made experiments with these forms of the same type as were conducted with *Mya arenaria* temporarily impossible. Neither of these forms is normally submitted to the same degree of low oxygen tension as is *Mya arenaria*. It is hoped that experimental work of a similar nature to that carried out with *Mya arenaria* may be done on other forms at a more favorable time of the year.

It has long been known that animals show a very unequal resistance to lack of air. Bunge (11) in his work upon respiration of intestinal parasites and mud-dwelling organisms showed that parasites in the intestine of warm blooded animals must live practically in the absence of oxygen, while worms living in the mud were also subject to similar conditions, decomposition processes, with the formation of reducing substances, keeping the oxygen absent. Packard (12) found that worms and mud-dwelling Crustacea are resistant to the lack of oxygen for some time.

The ability of an animal to resist a lack of oxygen may or may not be connected with an anaerobic respiratory mechanism. If one finds complete evidence of metabolism in an animal exposed to anaerobic conditions, then anaerobic respiration would be indicated. Such seems to be the condition in the case of *Mya arenaria*. Crustacean types which were exposed to the air or submerged in boiled sea water died within a few hours. The carbon dioxide content of the blood was, however, practically unaltered by such procedures. These forms do not use the calcium carbonate of their carapace as a protective measure when removed from their normal habitat.

In the light of the results of the experiments which have so far been conducted upon *Mya arenaria* the writer has tentatively to conclude that individuals of this species behave as facultative anaerobic organisms. It is realized, however, that in these preliminary experiments absolutely anaerobic conditions were not secured. It is the intention to continue this work at another time when it is hoped to determine the hydrogen ion concentration of the celomic fluid and the rate of oxygen consumption under various conditions.

SUMMARY.

1. Calcareous shelled pelecypod Mollusca and the arthropod *Balanus aquilla* have in the calcium carbonate of their shells a potentially great alkali reserve.

2. Exposure of these forms to atmospheric air causes a marked increase in the combined carbon dioxide of the celomic fluid.

3. There is under these circumstances a parallel increase in the calcium concentration and the buffer value of the celomic fluid.

4. Various other marine forms studied did not so react.

5. There is no increase in the total nitrogen of the celomic fluid of the pelecypod Mollusca exposed to atmospheric air.

6. *Mya arenaria* is particularly resistant to long exposure to atmospheric air.

7. When specimens of *Mya arenaria* are placed in a relatively small volume of fresh sea water, boiled sea water, distilled water, or in a hydrogen or a nitrogen atmosphere much the same reaction is observed as when specimens are exposed to atmospheric air.

8. The rate of increase in the content of carbon dioxide, the calcium concentration, and the buffer value of the celomic fluid under all the above conditions is, during the first period of several hours, constant.

9. The rate of increase in the rate of combined carbon dioxide, the concentration of calcium, and the buffer value is not so great in a hydrogen or nitrogen atmosphere as it is in air.

10. It is suggested that *Mya arenaria* is a facultative anaerobic organism which continues to produce carbon dioxide under anaerobic conditions.

In conclusion I wish to express my thanks to Dr. C. McLean Fraser, the Curator of the Biological Station, Departure Bay, British Columbia, for his cooperation during the carrying out of the experimental work reported in this communication.

My thanks are also due to the Biological Board of Canada, by whom the expenses in connection with this investigation have been defrayed.

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RELATION OF THE ALKALI RESERVE OF THE BLOOD TO GLYCOSURIA AND HYPERGLYCEMIA IN PANCREATIC DIABETES.

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The failure of the body to utilize carbohydrate and the increased combustion of fat in diabetes are regarded by most workers as responsible for the production of an excessive amount of acids, particularly aceto-acetic and β-hydroxybutyric acid, which cause a diminution of the alkali reserve of the blood and presumably of the tissues. It has been shown, on the other hand, by Elias (1912–13), and Elias and Kolb (1913), and others,[1] that the administration of mineral acids to dogs causes glucosuria and hyperglycemia. Jensen's (1918) work, however, seems to indicate that if the acid is sufficiently dilute much larger quantities may be given to dogs without causing an excretion of glucose by the kidney. He was able to increase glucosuria in dogs by administering 600 cc. of 0.1 N hydrochloric acid solution, only after seven-eighths of the pancreas had been removed.

Murlin and Kramer (1916) were able to increase the amount of glucose in the blood and urine of depancreatized dogs by giving, by mouth, a solution of hydrochloric acid. These results led Murlin and Sweet (1916–17) to study the course of pancreatic diabetes after the extirpation of the stomach. They found a much milder form of diabetes appeared when both stomach and pancreas were removed, than when the pancreas alone was extirpated. They believe their results indicate that the influence of the pancreas upon carbohydrate metabolism consists, at least in part, in neutralizing the acid produced by the stomach, thereby

[1] The literature on the effects of administering acids to animals is reviewed in the papers of Elias and of Elias and Kolb.

protecting the liver from acid intoxication. Their theory of the function of the pancreas is as follows:

"The results of this study . . . . suggest that the internal function of the pancreas may be closely akin to its external function in that, on the one hand (external), it provides for the complete neutralization of the acid contents of the stomach, thereby protecting the liver in its glycogenic function, and on the other (internal), it somehow preserves the proper concentration of hydrogen ions in the tissues for the combustion of glucose. It is not impossible that the pancreatic hormone, through which the latter function is discharged, may prove to be a peculiarly adapted alkali produced by the islands of Langerhans."

EXPERIMENTAL.

If the gastric juice were not completely neutralized in the intestine after the removal of the pancreas, it would react with the carbonates and phosphates of the blood in the portal system causing a reduction in the alkali reserve in the whole blood, inasmuch as the blood from the portal system is mixed with the blood from the rest of the body in the heart, lungs, and, to a less extent, in the liver. Since hyperglycemia and glucosuria appear very soon after the removal of the pancreas, it was thought worth while to study the alkali reserve of the blood as measured by the power of the plasma to combine with carbon dioxide, and compare this with the change in the concentration of glucose in the blood, and its excretion into the urine. The capacity of the plasma for carbon dioxide was determined by the method of Van Slyke (1917), Maclean's (1919) method for blood sugar was used, and the glucose in the urine was determined by titrating against Fehling's solution. Dr. Sweet removed the pancreas from the dogs by his usual technique.

DISCUSSION.

Our results from five dogs are given in Table I. We found that the alkali reserve of the blood, as measured by the carbon dioxide capacity of the plasma, showed no decrease until diabetes, as shown by the G:N ratio and increase in blood sugar, had been established for at least 24 hours. The earliest appearance of a decrease in the carbon dioxide capacity of the blood came about

TABLE I.

Time after operation.	CO_2 per 100 cc. plasma.	Glucose.*	Glucose in urine.	Nitrogen in urine.	G:N	Remarks.
			No. 1. Pregnant female.			
days	*cc.*	*per cent*	*gm.*	*gm.*		
Before.	41.5	0.063				
1	56.1	0.1998	17.70	7.18	2.47	Fasted.
2	59.6	0.257	18.93	8.59	2.2	
3	51.5	0.321	24.62	9.41	2.62	
			No. 2. Female.			
Before.	50.4	0.059				Fasted.
1	54.6	0.177	Urine lost.			
2	44.2	0.161	30.66	10.83	2.83	Urine may not have been properly preserved.
3	48.9	0.163	19.3	6.87	2.83	Animal died during the night of 4th day after operation.
			No. 3. Male.			
Before.	45.3	0.055				Fasted.
1	47.6	0.118	15.93	6.71	2.37	
2	56.3	0.119	28.64	10.4	2.75	
3	42.2	0.143	23.52	9.2	2.53	No evidence of peritonitis found.
6	30.9	0.120	22.6	7.37	3.07	Found dead on 7th day.
			No. 4. Male.			
Before.	51.2	0.073				Urine not preserved.
1	58.0	0.150				
2	41.5	0.123	23.8	9.45	2.52	
3	42.0	0.148	17.09	9.04	2.33	
4	41.1	0.243				Animal fed ½ lb. meat per day. Refused food on 4th day. Found dead on 5th day. No evidence of peritonitis was found.

* Computed on whole blood.

TABLE I—*Concluded.*

Time after operation.	CO_2 per 100 cc. plasma.	Glucose.*	Glucose in urine.	Nitrogen in urine.	G:N	Remarks.
			No. 5. Female.			
days	*cc.*	*per cent*	*gm.*	*gm.*		
Before.	51.7	0.075				
1	51.4	0.098	33.91	15.19	2.23	Urine of 1st and 2nd days.
2	52.8	0.293				
3	49.9	0.241	24.19	9.64	2.51	Animal fed ½ lb. meat per day.
4	39.5	0.270	26.18	8.69	3.01	
5	39.6	0.238	17.34	8.47	2.05	
7	38.9	0.270	16.17	7.31	2.21	
8	34.7	0.280	16.46	7.20	2.28	
9	29.8	0.315	24.52	10.05	2.44	Killed.

48 hours after the removal of the pancreas, while there was always a definite increase in the blood glucose within 1 day after the operation. It is well known that glucose appears in the urine within a few hours after pancreatectomy. It is of interest to note the more or less marked increase (1 to 20 per cent by volume of CO_2) in the alkali reserve on the 1st or 2nd day after the operation. In most cases, this increase is comparatively small and its significance is not clear.

It would seem that if acid is taken up from the intestine by the portal blood after the removal of the pancreas, it must be neutralized by some base other than bicarbonates or phosphates of the blood, or cause a simultaneous liberation of alkali from some part of the body. It may be pointed out in this connection that the formation of a pancreatic fistula, so that the pancreatic juice escapes to the exterior, does not cause any disturbance in the carbohydrate metabolism of the animal. In such cases, the acid from the gastric juice would be as likely to reach the liver as when the pancreas had been removed from the body. It is questionable, also, whether fragments of the pancreas, which are sufficient to prevent diabetes, can produce enough pancreatic juice to be of any importance in neutralizing the acid produced in the stomach.

Acidosis in depancreatized dogs undoubtedly develops as shown by Nos. 3 and 5. In No. 3, the carbon dioxide capacity of the plasma fell to about 31 volumes per cent, and in No. 5, to about 30 volumes per cent.

SUMMARY.

1. A decrease in the alkali reserve of the blood, as shown by the carbon dioxide capacity of the plasma, is not simultaneous with the appearance of hyperglycemia and glucosuria in pancreatectomized dogs.

2. A slight increase in the carbon dioxide capacity of blood plasma was observed on the 1st or 2nd day after the removal of the pancreas, later decreasing and gradually reaching about 30 volumes per cent in two cases.

3. Definite acidosis appears comparatively late in pancreatic diabetes in dogs.

4. No observations which support Murlin and Sweet's theory of the causal relation of the acid of the gastric juice to pancreatic diabetes were made.

We wish to take this opportunity to thank Dr. J. E. Sweet very kindly for doing the surgical work.

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THE GLOBULIN OF THE COHUNE NUT, ATTALEA COHUNE.

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Plate 1.

(Received for publication, September 30, 1920.)

The cohune palm (1, 2) is indigenous to the British Honduras (3) and the other countries of the Caribbean coastal region, and its nut is shown whole, opened, and in cross-section in Fig. 1.[1] Most of the nuts have but one kernel, a few two.

It is found mostly in damp lands, growing best in the deep forest (4). The nuts are produced in large clusters of about 75 pounds each, a single tree having an average yearly production of four clusters. They vary in size from $1\frac{1}{2}$ to 3 inches in length and from 1 to 2 inches in diameter, the shell being hard and dense with an average thickness of $\frac{1}{4}$ to $\frac{1}{2}$ inch. In certain districts the cohune palm constitutes 20 to 30 per cent of the vegetation (3). The kernel averages about 10 per cent by weight of the whole nut.

The nut was not commercially exploited until the World War when the shells were carbonized and used in gas masks, the kernels being pressed for their oil, which is of a very high quality and is similar to coconut oil (3–5).

A search has not yet disclosed literature bearing on the utilization of the press-cake. The nuts are now being pressed for oil in the British Honduras, and experiments are in progress in this country to produce the oil on a commercial scale. The press-cake will be suitable for use in feeds, its value as such being indicated by the analysis of the isolated protein described herein.

[1] This information and photograph were furnished by Mr. O. F. Cook of the Office of Crop Acclimatization, United States Department of Agriculture, Washington.

TABLE I.

Comparison of Analysis of Coconut and Cohune Nut Globulins.

Moisture- and ash-free basis.

	Coconut globulin.		Cohune nut globulin.	Distribution of nitrogen.‡		
	*	†		N	Coconut globulin.‡	Cohune nut globulin.
	per cent	*per cent*	*per cent*		*per cent*	*per cent*
C	50.88	51.23	51.75	Amide	1.36	1.35
H	6.82	6.90	6.92	Humin	0.14	0.17
N	17.89	18.40	17.82	Basic	6.06	7.14
S	1.03	1.06	1.35	Non-basic	10.92	9.34
O§	23.38	22.41	22.16			
Ash	0.98	0.25	1.83			

* Ritthausen (6).
† Chittenden (7).
‡ Osborne and Harris (8).
§ Calculated by difference.

TABLE II.

Comparison of Analysis of Coconut and Cohune Nut Globulins.

Analyses by the Van Slyke method. Total N regained corrected for solubility of bases (9).

	Coconut globulin.*	Cohune nut globulin.
	per cent	*per cent*
Amide N	7.99	7.50
Humin N adsorbed by lime	1.41	0.84
" N in amyl alcohol extract	0.11	0.11
Cystine N	0.96	0.53
Arginine N	29.50	30.87
Histidine N	3.68	2.61
Lysine N	6.41	7.94
Amino N of filtrate	45.44	47.87
Non-amino N of filtrate	4.60	2.28
Total N regained	100.10	100.55
Distribution of the basic amino-acids.		
Cystine	1.44	0.81
Arginine	15.92	17.17
Histidine	2.42	1.72
Lysine	5.80	7.42
Tryptophane	Present.	Present.

* Johns, Finks, and Gersdorff (10).

This analysis shows that the protein contains all the basic amino-acids necessary to produce normal growth. The protein also gives a strong qualitative test for tryptophane.

The press-cake used in this investigation was prepared in this Laboratory from nuts furnished by the United States Food Administration. It contained 20.63 per cent of protein (N × 6.25).

The principal protein extracted by means of sodium chloride solution is a globulin. This was obtained by dialyzing the saline extracts and could also be precipitated from the extracts by means of ammonium sulfate. This globulin is quite similar to that obtained from the coconut, no marked differences being observed in any of the analyses. These comparative results are given in Tables I and II.

Tests for albumin showed that a trace was present. This albumin coagulates between 60° and 68°C. while the globulin coagulates at near the boiling point of water.

EXPERIMENTAL.

Preparation of the Press-Cake.—Much difficulty was experienced in removing the husk and shell. This was accomplished by cracking each nut individually between a steel plate and the head of a steel power drill. The kernels which ran mostly one to a nut, seldom two, were cold-pressed, using a press of the oil expeller type. The granular press-cake was freed from residual oil by extracting twice with petroleum ether, the excess of ether being removed by drying *in vacuo* over sulfuric acid. The press-cake was then ground to a fine meal which contained 20.63 per cent of protein (N × 6.25).

Preliminary Experiments.—Extraction experiments were made with different concentrations of sodium chloride in water, with 70 per cent alcohol and with 1 per cent hydrochloric acid, extracting for 1 hour in each case. The percentages of protein extracted by the various solvents are given in Table III. Time extractions were then made with the solvent which extracted the maximum quantity of protein, and the data are given in Table IV.

All extractions described in this paper were made with a 10 per cent sodium chloride solution in distilled water. This solvent extracted the maximum quantity of protein, 16.09 per cent, in 1 hour.

TABLE III.

Preliminary Extraction Experiments.

Solvent to 1 gm. of meal.	Solvent.	Protein extracted (N × 6.25).
cc.		*per cent*
10	70 per cent alcohol.	0.52
10	1 " " HCl.	12.66
10	Distilled water.	4.04
10	0.5 per cent NaCl.	7.74
10	1.0 " " NaCl.	11.52
10	1.5 " " NaCl.	11.08
10	2.0 " " NaCl.	12.39
10	2.5 " " NaCl.	14.51
10	3.0 " " NaCl.	14.59
10	3.5 " " NaCl.	15.13
10	4.0 " " NaCl.	15.03
10	5.0 " " NaCl.	15.21
10	7.0 " " NaCl.	15.30
10	8.5 " " NaCl.	15.91
10	10.0 " " NaCl.	16.09

TABLE IV.

Time Extraction Experiments.

Solvent: 10 per cent sodium chloride solution, 10 cc. to 1 gm. of meal.

Time extracted, *hrs*	½	1	2	3	20	45	69
Protein extracted (N × 6.25), *per cent*	13.71	16.09	14.16	14.07	13.71	13.14	12.84

TABLE V.

Globulin Precipitated by 0.7 of Saturation with Ammonium Sulfate.

Calculated on moisture- and ash-free basis.

	I	II	Average.
	per cent	*per cent*	*per cent*
C	52.77	52.62	52.70
H	7.03	7.06	7.05
N	17.80	17.68	17.74
S	1.21		1.21
O*			21.30
Moisture		6.87	
Ash		0.53	

* Calculated by difference.

Globulin Extracted by Sodium Chloride and Precipitated by Ammonium Sulfate.—An extraction was made, using 10 cc. of solvent to each gram of meal. The quantity of meal used for a preparation ranged from 125 to 210 gm. The meal was mixed with the solvent, all lumps being disintegrated by hand, and the mixture allowed to stand at room temperature for 1 hour. The mixture was then poured into a large Buchner funnel fitted with a circular piece of closely woven cotton cloth, and the extract recovered by suction, the residue being washed twice with 10 per cent sodium chloride solution. The extract so recovered was nearly clear and was easily filtered through a mat of paper pulp on a Buchner funnel. The extract was then dialyzed in parchment bags suspended in running chilled water until practically free from chlorides. The dialyzed protein was washed and then redissolved in a volume of 10 per cent sodium chloride solution equal to about one-third the volume of the solvent originally used in extracting the meal. It was filtered clear through a mat of paper pulp on a Buchner funnel. The clear extract was made 0.7 saturated with ammonium sulfate and allowed to stand over night. The precipitated protein was filtered on a folded soft filter paper, redissolved in distilled water, filtered clear, and dialyzed for 12 days. The protein was removed from the mother liquor in the dialyzing bags, washed free from chlorides and sulfates, and dried. The water was removed by treating the protein with absolute alcohol, removing the alcohol by suspension in absolute ether, filtering, and drying, first over sulfuric acid *in vacuo* and then in a vacuum drying oven, gradually raising the temperature to 110°C. It was then ground to a fine powder in a mortar, the finished product being an amorphous white powder. The yield based on the weight of the meal used was 5.6 per cent. In order to facilitate the weighing of samples, all preparations of protein were exposed to the air to absorb moisture until an equilibrium was reached.

The analyses of this preparation, calculated on a moisture- and ash-free basis, are given in Table V.

Globulin Extracted by Sodium Chloride and Fractionally Precipitated by Ammonium Sulfate.—A sodium chloride extract was made as described above and the clear solution was gradually made from 0.1 to 0.7 saturated with ammonium sulfate, first

experimenting with a small portion of the extract. No precipitation occurred until the 0.3 saturation point was reached, and then only a slight turbidity was obtained which very gradually increased on adding each successive fraction of ammonium sulfate to make the solution 0.05 more saturated, until the 0.5 saturation point was reached, when the precipitate became flocculent after standing 18 hours. This precipitate was filtered off and washed several times with 10 per cent sodium chloride solution made 0.5 saturated with ammonium sulfate. It was redissolved in 10 per cent sodium chloride solution, filtered and dialyzed for 12 days, washed, and dried as previously described. This is Preparation II. The yield based on the meal used was 3.2 per cent.

The 0.5 saturated ammonium sulfate filtrate was then made successively 0.05 more saturated until the 0.7 of saturation was reached, after which no more precipitate was obtained. A slight turbidity occurred at 0.55 of saturation which increased as each successive fraction was added, becoming flocculent at 0.7 of saturation. This fraction was filtered on a folded filter paper and redissolved in distilled water. It was filtered clear and dialyzed for 12 days. The dialyzed protein was washed and dried as previously described. The yield based on the meal used was 1.6 per cent. This is Preparation III.

The analyses of Preparations II and III calculated on the moisture- and ash-free basis appear in Table VI.

We were unable to fix on a decisive separation point and are inclined to believe that but one globulin is present in the cohune nut. Our belief is strengthened by the analyses of these two fractions as compared with the analyses of the globulin obtained in Preparations I, IV, and V, the same difference in carbon content being evident in the preparations of the globulin as appears in the two preparations made by fractional precipitation with ammonium sulfate. We were unable to make a further study along this line owing to a lack of material.

Globulin Extracted by Sodium Chloride and Precipitated by Direct Dialysis.—Two sodium chloride extracts were made as before, and the clear extract was dialyzed in parchment bags against running chilled water for 12 days. The dialyzed protein was washed and dried as described previously in this paper. The

yield of protein in each case was 8.57 per cent, based on the meal used.

TABLE VI.

Fractional Precipitation of the Globulin by Ammonium Sulfate.

Calculated on moisture- and ash-free basis.

	Preparation II. 0.5 saturated ammonia sulfate precipitate.			Preparation III. 0.7 saturated ammonia sulfate precipitate.		
	I	II	Average.	I	II	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C	50.70	50.73	50.72	52.87	52.73	52.80
H	6.77	6.84	6.81	7.18	6.99	7.09
N	17.64	17.76	17.70	17.63	17.77	17.70
S	1.43		1.43	1.23		1.23
O*			23.34			21.18
Moisture		6.46			7.92	
Ash		1.00			0.90	

* Calculated by difference.

The analyses of these two preparations (Preparations IV and V) calculated on a moisture- and ash-free basis appear in Table VII.

TABLE VII.

Direct Dialysis Preparations.

Calculated on moisture- and ash-free basis.

	Preparation IV.			Preparation V.		
	I	II	Average.	I	II	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C	52.13	52.07	52.10	50.42	50.47	50.45
H	6.75	6.90	6.83	6.90	6.77	6.84
N	18.08	17.99	18.04	17.86	17.93	17.90
S	1.45		1.45	1.42		1.42
O*			21.58			23.39
Moisture		7.90			6.93	
Ash		3.65			3.05	

* Calculated by difference.

A summary of the analyses of the five preparations is given in Table VIII.

TABLE VIII.

Summary of the Analyses of the Cohune Nut Globulin.

Calculated on moisture- and ash-free basis.

	Preparation No.					Average.
	I	II	III	IV	V	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C	52.70	50.72	52.80	52.10	50.45	51.75
H	7.05	6.81	7.09	6.83	6.84	6.92
N	17.74	17.70	17.70	18.04	17.90	17.82
S	1.21	1.43	1.23	1.45	1.42	1.35
O*	21.30	23.34	21.18	21.58	23.39	22.16

* Calculated by difference.

TABLE IX.

Analyses by the Van Slyke Method.

Total N regained corrected for solubility of bases (9).

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0379	0.0367	7.47	7.53	7.50
Humin N adsorbed by lime	0.0042	0.0041	0.83	0.85	0.84
" N in amyl alcohol extract	0.0006	0.0004	0.12	0.09	0.11
Cystine N	0.0026	0.0026	0.51	0.54	0.53
Arginine N	0.1562	0.1506	30.77	30.96	30.87
Histidine N	0.0132	0.0127	2.60	2.61	2.61
Lysine N	0.0417	0.0373	8.21	7.66	7.94
Amino N of filtrate	0.2427	0.2331	47.81	47.92	47.87
Non-amino N of filtrate	0.0140	0.0088	2.75	1.81	2.28
Total N regained	0.5131	0.4863	101.07	99.97	100.55

TABLE X.

Distribution of Basic Amino-Acids in Cohune Nut Globulin.

	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	0.79	0.82	0.81
Arginine	17.12	17.22	17.17
Histidine	1.72	1.72	1.72
Lysine	7.67	7.16	7.42
Tryptophane			Present.

Analysis of the Globulin by the Van Slyke Method.—A globulin obtained by direct dialysis was analyzed in duplicate, some of the determinations on each sample also being done in duplicate. The samples weighed 3.1505 and 3.0190 gm. equivalent to 2.8361 and 2.7177 gm. of moisture- and ash-free samples, respectively, containing 17.90 per cent of nitrogen. Sample I contained 0.5077 gm. and Sample II contained 0.4865 gm. of nitrogen.

The protein was dissolved in 100 cc. of 20 per cent hydrochloric acid and hydrolyzed by boiling the solution under a condenser for 36 hours. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method (11). The results are given in Tables IX and X. The figures for cystine are undoubtedly low since this amino-acid is slowly decomposed in boiling hydrochloric acid.

TABLE XI.

Free Amino Nitrogen of Cohune Nut Globulin Compared with the Lysine Nitrogen.

Total N in 2 cc.	N gas from 2 cc.	Pressure.	Temperature.	Amino N in 2 cc.	Ratio of amino N to total N.	One-half lysine N by Van Slyke method.
mg.	*cc.*	*mm.*	*°C.*	*mg.*	*per cent*	*per cent*
13.28	0.75	761.2	24.0	0.419	3.16	3.97
13.28	0.83	761.2	24.5	0.463	3.48	

Free Amino Nitrogen of the Globulin Compared with the Lysine Nitrogen.—In order to determine the ratio between the free amino nitrogen of the unhydrolyzed globulin and the lysine nitrogen content as determined by the Van Slyke method, about 2 gm. of the wet globulin used in the Van Slyke analysis were dissolved in 0.1 N sodium hydroxide solution and made up to 25 cc. Two 2 cc. portions of the solution were used for the free amino nitrogen determinations, using Van Slyke's micro apparatus (12). Total nitrogen was determined on 5 cc. aliquots and the results were calculated to the basis of 2 cc. Diphenyl ether was used to prevent foaming and corrections were made for the reagents used. The free amino nitrogen found agrees fairly well with one-half the lysine nitrogen as determined by the Van Slyke method. The results will be found in Table XI.

Distribution of the Nitrogen in the Globulin.—The distribution of the nitrogen in the globulin was calculated from the results obtained by the Van Slyke method, the calculation being comparable with analyses obtained by Hausmann's modified method (8). The distribution of the nitrogen is shown in Table XII.

TABLE XII.

Distribution of Nitrogen in Cohune Nut Globulin.

N	Globulin precipitated by dialysis.		
	I	II	Average.
	per cent	*per cent*	*per cent*
Amide	1.34	1.35	1.35
Humin	0.17	0.17	0.17
Basic	7.18	7.10	7.14
Non-basic	9.41	9.27	9.34
Total	18.10	17.89	18.00

SUMMARY.

1. The globulin of the cohune nut has been isolated and analyzed, and a trace of albumin has been shown to be present in the nut.

2. The analyses of the globulin reveals a close similarity between it and the globulin of the coconut.

3. The basic amino-acids of the globulin were determined by the Van Slyke method and the distribution of the nitrogen (Hausmann's) was calculated from these results.

4. The analyses indicate that the cohune nut globulin contains all the basic amino-acids known to exist in proteins, being high in arginine and lysine. Tryptophane is also present.

5. The free amino nitrogen in the globulin has been determined and has been found to agree fairly well with one-half the lysine nitrogen as determined by the Van Slyke method.

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EXPLANATION OF PLATE 1.

FIG. 1. The cohune nut, *Attalea cohune* Martius. Natural size.

FIG. 1.

(Johns and Gersdorff: Globulin from the cohune nut.)

CONTRIBUTIONS TO THE BIOCHEMISTRY OF IODINE.

III. THE COMPARATIVE EFFECTS OF THYROID AND IODIDE FEEDING ON GROWTH IN WHITE RATS AND IN RABBITS.*

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INTRODUCTION.

This investigation was carried out during the winter and spring of 1919–20. It was commenced with the idea of determining definitely whether thyroid feeding had any effect on growth in young animals,[1] and the earlier experiments dealt only with this one problem, and the comparative effect of iodide. In subsequent experiments observations were also made on the degree of hypertrophy produced in certain organs.

E. R. Hoskins (1916) has summarized the earlier literature relating to both problems. In determining the effect on gross weight the majority of earlier observations are on adult animals only. The following refer to growth in young animals:

Moussu (1899) found that small doses of thyroid stimulate the rate of growth in young dogs, but that large doses are toxic. Bircher (1910) fed thyroid to young rats, and obtained a retardation in growth and body weight, but an acceleration in the process of ossification. Schafer (1912) noted in young white rats fed thyroid gland an increased food consumption, increased metabolism, and acceleration of growth. Fordyce (1912) observed a decrease in rate of growth. Gudernatsch (1912–13, 1913–14) found that administration of thyroid to tadpoles retarded growth, but hastened metamorphosis.

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* A preliminary note, outlining the results arrived at in this investigation, has been communicated to the Physiological Society (England).

[1] Some preliminary and not very exact experiments by A. T. Cameron and T. D. Wheeler in this laboratory in 1914–15 had not yielded definite results.

Hoskins himself used white rats for his experiments. His results, based on average figures for a number of rats, do not show any definite effect of thyroid feeding on the rate of growth; the growth curves were approximately the same as those from the average figures for a series of controls from the same litters. He pointed out that the normal growth of the albino rat shows material variation, not only in different strains and under different conditions of environment, but even among litters when all conditions are kept as nearly constant as possible, so that in all such tests experimental and control animals should be selected of the same sex and from the same litter. He used both fresh gland and recently desiccated gland without any noticeable difference in result. The dose given every 2nd day varied from 10 mg. of desiccated (or 40 mg. of fresh) to 200 mg. of desiccated calf thyroid gland in different experiments.

Herring (1917, *b*) used white rats, carefully comparing thyroid-fed and control animals from the same litter and of the same sex. Fresh ox thyroid (0.1 to 0.2 gm.) was fed daily. A slight decrease was observed in the rate of growth of male rats. With females, more variable results were obtained. They appeared to be more susceptible to the treatment, but the average gain in weight differed very little from that of the controls (*cf.* also Herring (1919)).

Hewitt (1920) has just published the results of two series of similar experiments. One was on adult rats. In the second he claims to have obtained a decrease in growth rate, but gives no details of experimental observations on controls, although he indicates that such were made.

(Compare also the results of Iscovesco with a specific preparation, noted below.)

The discrepant results just recorded are in our opinion due to two sources of error: the first, non-comparison of every experimental animal with a control from the same litter kept rigidly under the same conditions (this source of error was excluded by Herring); the second, the variability of dosage. It seems natural to infer that a fixed dose, given over a long period to a rat of constantly changing weight, and to a series of rats of different initial weights, would not produce comparable effects; this method of dosage has been employed, however, by almost all the investigators of this problem. We have used a dose based rigidly on and bearing a constant ratio to the body weight of the animal at the time of administration, and have achieved concordant results.

The literature concerned with the second problem will now be outlined.

R. G. Hoskins (1910) fed daily for 15 days varying amounts (5 to 15 mg.) of thyroid to eighteen young guinea pigs. The adrenal glands averaged 25 per cent heavier than those of the same number of controls. Iscovesco (1913), using a lipoid preparation from thyroid (the part of the petroleum-ether extract insoluble in acetone), and experimenting with young rabbits, claims to have obtained a marked acceleration of general growth with young animals (initial age 45 days), and a slight decrease in growth rate with older animals (initial age 4½ months). 2 months treatment with a dose of this preparation of 0.01 to 0.02 gm. per kilo of animal per day caused marked hypertrophy of adrenals, spleen, thyroid, testes, uterus and ovaries, a distinct effect on the heart and kidneys (the latter in females only), and no effect on the liver. His results are not in good agreement with those of subsequent workers, and Fenger's (1916) work on the phosphatide content of thyroid throws doubt on the lipoid nature of his preparation.

E. R. Hoskins (1916), in the experiments already described, found that thyroid feeding produced a decided hypertrophy of the heart, liver, spleen, and adrenal glands (especially in males), a somewhat more uncertain and less extensive increase in the weight of the alimentary canal and pituitary (male), and possibly in the skeleton, testes, and epididymi, and a decrease in the weight of the (female) pituitary. On the other hand, feeding with thymus, pineal, or pituitary produced no marked or constant change upon the growth rate of the body or organs.

Similarly R. G. and A. D. Hoskins (1916) found that the only effect of this nature from feeding adrenals to white rats was hypertrophy of testes, and in some cases hypertrophy of ovaries.

Kojima (1916) found that the pancreas hypertrophies; he described the occurrence of numerous mitotic figures in the nuclei and alterations in the granules of the cells.

Herring generally confirms Hoskins' results. Thyroid feeding produces enlargement of the adrenals, heart, liver, spleen, testes, and ovaries, no constant change in the thymus, and a decrease in size of the (female) pituitary. The pancreas is enlarged (confirming Kojima), while the thyroid glands are decreased in weight and show signs of relative inactivity. Herring especially draws attention to the marked hypertrophy which may occur with heart and kidneys. While Hoskins thought that the hypertrophies may be ascribable to the general increase in metabolism in the body, Herring takes the view that the adrenin output is compensatory to the thyroid, more especially in its action on the circulation, and the combination of these factors along with the increased metabolism may be the cause of the great hypertrophy of heart and kidneys.

Herring (1915–16, 1917, *a*) had previously shown that the adrenin content of the adrenals was increased. Kuriyama (1918) was unable to find an increase either in the size of the gland or in the adrenin content. Hewitt (1920) criticizes his method, and confirms Hoskins and Herring with respect to the adrenals, heart, liver, and spleen, and Herring with respect to the diminution in size of the thyroid. He finds that the pitui-

tary hypertrophies in the majority of cases (male rats). He uses for comparison figures Donaldson's (1915) collected data, a method open to some objection, as Hoskins has pointed out. He considers further that his results indicate that the abnormal condition is probably temporary, and that a return, perhaps incomplete, to normal proportions is indicated after cessation of thyroid feeding.

The loss of fat as a result of thyroid feeding is well known. Both Hoskins and Herring noted it in all their experiments, and this loss was considered to balance the organ hypertrophy and so mask any total weight changes.

Cramer and Krause (1912–13) found that, if cats and rats were fed thyroid, in 2 or 3 days glycogen completely disappeared from the liver, even on a rich carbohydrate diet, and this observation has been confirmed by Kuriyama and others. Kuriyama was unable to observe this loss of glycogen in the frog.

We are unaware of any experiments with iodides similar to those described above with thyroid.

It has been repeatedly shown that the iodine content of the thyroid is easily influenced by iodine in the diet, whether fed as inorganic or organic iodine, or as thyroid itself.[2] Fordyce showed that after thyroid feeding the vesicles of the thyroid of the rat were distended with colloid. Kojima (1917) obtained this result both after administration of thyroid and of sodium or potassium iodide. The epithelial cells lining the vesicles are flattened. Herring's findings are not exactly in agreement.

EXPERIMENTAL RESULTS.

The animals employed were white rats and rabbits. In all, forty male and seventeen female rats were used. Of these, thirty-one males, and sixteen females were from nine litters; seven controlled experiments were carried out with thyroid, and four with sodium iodide. The initial age of feeding varied from 40 to 60 days. The remaining ten animals were of different ages and from different litters, and were used in a specific experiment to induce tetany by feeding large thyroid doses. Most of the litters were born and reared in the Department; some were purchased locally when still quite young.

Previous to treatment, the rats were isolated in small cages, groups of such cages being kept side by side. The rats were weighed to the nearest 0.5 gm. each morning before food was given. Except in the first experiment, unlimited bread and

[2] See, for example, Baumann and Goldmann (1896), Roos (1899), and Simpson and Hunter (1911).

milk were fed throughout. The dried thyroid was weighed to the nearest milligram, mixed with flour (except where indicated), made up into a thick paste with water, and fed on a watch-glass before other food and immediately after weighing. After the 1st day it was always eaten greedily and completely, so that the error from wastage can only amount to a small per cent. The dosage was, in different experiments, 1 mg. of dried thyroid to 20, 10, 5, and 2 gm. of the actual body weight at the time of feeding (1:20,000; 1:10,000; 1:5,000; 1:2,000).

Three thyroid preparations were used: the first a Merck (Darmstadt) preparation at least 9 years old, containing 0.39 per cent of iodine (termed below Thyroid A); the second a hog thyroid preparation containing 0.34 per cent of iodine (termed Thyroid B); and the third a sheep thyroid preparation containing 0.18 per cent of iodine (termed Thyroid C). The two latter were prepared in December, 1919, and were obtained from the Armour laboratories through the kindness of Dr. F. Fenger.

In order to control any toxic effects of the thyroid preparations, not due to the specific nature of the tissue, ox liver, purchased locally, and dried at 100°C. without any previous aseptic or antiseptic precautions, was fed in amounts equal to the thyroid dose in a number of experiments, until it became evident that no different effect was observable with these controls, and with controls fed solely on bread and milk.

Sodium iodide was used on account of the non-activity of the sodium ion. Dilute solutions were employed of such strength that not more than 1 or 2 cc. were required to give the requisite dose. This was measured with a graduated pipette, mixed with flour to a thick paste, and given on a watch-glass as usual.

At the conclusion of each experiment the animals were killed with chloroform, the organs dissected, the larger weighed immediately, and the smaller transferred to closed weighed glass vessels for subsequent weighing. Where indicated below, one thyroid lobe was transferred to corrosive sublimate, and after hardening was sectioned and stained with hematoxylin-eosin for histological examination. The other lobe, or the whole thyroid, was dried at 100°C. for dry weight determinations. Attempts to estimate the small amounts of iodine in the single lobes by the Kendall (1914) method were not very successful and were abandoned.

In some of the later experiments Kendall's recent modification (1920) was used with successful results.

The tables show the body weights on every 3rd day. The daily weighings give somewhat less regular curves, probably largely owing to room temperature variations from the extreme climatic conditions. Since these were the same for control as for treated rats, they can be neglected.

In a few cases, where thyroid-fed rats died suddenly, and the controls were being used for other treated rats from the same litter, organ weights are given in the tables for comparison, taken from Donaldson's averages.

In one experiment five rabbits from one litter, born in the Department, were used. They were isolated into separate cages, handled, and weighed daily for some time before treatment commenced, as it was found that initially this handling appeared to affect them considerably. Their diet consisted of unlimited oats, hay, and green stuff. When their growth rate became relatively constant they were fed each morning dry thyroid mixed with dry ground oats, immediately after weighing and before other food was given. They soon became habituated to this treatment, and the thyroid was consumed with very little loss. At the end of the experiment they were killed and examined in the same way as the rats.

Experiment 1.—Thyroid A; 1:20,000; rats. Litter of four rats, two male and two female, born August 12th, 1919. Previously to the 39th day the diet consisted of oatmeal and household scraps. On the 39th day thyroid was fed with ground oatmeal to one male and one female, and the diet of all four was restricted to oatmeal and water. The diet showed a vitamine deficiency. On the 45th day green food was given in addition, and on the 49th day the diet was changed to bread and milk and 20 mg. of oatmeal in all cases. The control male rat was fed an amount of dried meat equal to thyroid from the 39th to the 70th days, and subsequently dried powdered ox liver. The control female rat was fed only the 20 mg. of oatmeal corresponding to that mixed with thyroid, in addition to the bread and milk. The figures for body weight are given in Table I.

Experiment 2.—Thyroid A; 1:20,000; rats. A litter of eight, five male and three female, born September 12th, 1919. Diet, unlimited bread and milk. Thyroid feeding was commenced on the 39th day. Two male controls were fed on liver corresponding in amount (1:20,000), and one male and one female control were given 20 mg. of oatmeal. All thyroid and liver doses were given with 20 mg. of oatmeal. The body weights are given in Table II.

The figures for dry thyroid in parentheses were calculated from those found for a single lobe. No. 1 was found dead on the 69th day. Macroscopic examination showed no obvious cause.

TABLE I.

Age.	Male.		Female.	
	Thyroid-fed.	Control.	Thyroid-fed.	Control.
days	*gm.*	*gm.*	*gm.*	*gm.*
39	60.5	65	55	55.5
42	51.5	62	52	55.5
45	54.5	61.5	49.5	53.5
48	54.5	60.5	50.5	56
51	61	76	57	66
54	75.5	80	67	76.5
57	84	82.5	70	81
60	92.5	95	73.5	80
63	96.5	98.5	80	86.5
66	102	113.5	85	90
69	104	118	89	96
72	108.5	125	88	102
75	111	130	96	102.5
78	116	137	90	104.5
81	124	144.5	95.5	108
84	126	143.5	105	111.5
87	136.5	148	103.5	116
90	146	158	114.5	117
93	144	166.5	113	121
96	148	169	115	125.5
99	153	171	120	129
102	156.5	177	126	133.5
105	159	186	134.5	135
108	162	190	136	135.5
111	163	191	136	132
114	174	194	144	135
115	170	198	146	139
Weight increase.				
In 18 days	23.5	17.5	15	25.5
" 36 "	50.5	65	41	47
" 72 "	102.5	126	81	76.5
Thyroid, fresh	0.0099	0.0203	0.0100	0.0126
" dry	0.0035	0.0044	0.0031	0.0028

TABLE II.

Age	Male.					Female.		
	No. 1. Thyroid.	No. 2. Thyroid.	No. 3. Liver.	No. 4. Liver.	No. 5. Oatmeal.	No. 6. Thyroid.	No. 7. Thyroid.	No. 8. Oatmeal.
days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
36	49	48.5	48.5	49	51.5	49	45.5	45.5
39	57	58.5	56.5	60	62.5	55	53	54.5
42	66	69.5	69.5	75	73.5	62	62.5	63.5
45	76.5	76.5	77.5	85	81.5	66.5	76	71
48	80	77.5	82	94.5	92	70.5	78	79
51	85	82	89.5	101.5	100.5	78	81.5	86
54	88	86	97	109	107	80	81	91
57	97	91	102.5	119	115	89	89	97
60	99.5	91	111.5	125.5	123.5	93.5	90	103.5
63	106	97	120	133	129	96	96	106.5
66	115	101	123	140	139.5	103	102	114
69	112	102.5	129	146.5	144	106.5	104	120
72		106	133.5	152	151	110	112	125
75		116	144	162	159	118	116	129
78		122.5	148	172	163.5	121	122	133
81		131	157	177.5	168.5	126.5	124	135
84		142	163	181	175	131	128	139.5
87		142	171	188	183	131	131	146
90		150	178.5	192.5	183	138.5	136.5	147.5
93		161	182	199	189	141	144	148
96		161	188	202.5	196	141.5	143	145
99		160	190	208	201	144	145	157.5

102		170	192	212	199	146	146	157	
105		168	197	222	207	149	151	155	
108		170	202	224	215	155.5	156	156	
111		168	207	226	215	154	156	160	
114		168	207	226	218	159	159	158	
115		166	212	232	219	157.5	159	161	
Weight increase.									
In 18 days	40	32.5	46	59	52.5	34	36	42.5	
" 36 "		57.5	87.5	102	96.5	63	63	74.5	
" 72 "		109.5	150.5	166	152.5	99	103	105.5	
Thyroid, fresh		0.0130	0.0178		0.0190	0.0121	0.0151	0.0144	
" dry		(0.0044)	(0.0050)		0.0046	(0.0034)	0.0042	(0.0043)	

Nos. 6 and 8 at the end of the experiment were killed, minced, and an ether extract was made. No. 6 yielded 8.5 and No. 8 14 gm. of ether-soluble material.

Experiment 3.—Thyroid A; 1:10,000; rats. A litter of two female rats, born October 11th, 1919. Thyroid fed with 20 mg. of oatmeal commencing on the 39th day; control animal fed 20 mg. of oatmeal. The body weights are given in Table III.

TABLE III.

Age.	Female. Thyroid-fed.	Female. Control.
days	*gm.*	*gm.*
36	45	45.5
39	53	53
42	55	57
45	60.5	67
48	63	70
51	68	75.5
54	71	78
57	74	84
60	76	88.5
63	88.5	94.5
66	95	101.5
69	100	107
72	99	108.5
75	103	110
78	107	118
79	103	119
Weight increase.		
In 18 days	21	31
" 36 "	50	57
Thyroid, fresh	0.0086	0.0087
" dry	0.0024	0.0022

Experiment 4.—Thyroid A; comparison of different dosages. A litter of three males and three females, born November 16th, 1919. Thyroid (mixed with flour) was fed from the 58th day. The controls were fed on bread and milk only. The body weights are given in Table IV and the organ weights in Table V.

The male rat fed a large thyroid dose was found dead on the 76th day (18th day of treatment). No definite cause was apparent. The female rat fed similar dose was found dead on the 80th day (22nd day of treatment). The cecum contained a small pebble. The rat had escaped from its cage for a few hours 3 days previously.

Experiment 5.—Comparison of different thyroid preparations. The litter consisted of five males and two females, born on January 5th, 1920. The females were used for iodide tests (see below). All thyroid doses were one-five thousandth of body weight, and were fed with flour. One control rat was fed equivalent amounts of dried liver, also given with flour. A second control was given bread and milk only. Treatment was commenced on the 46th day. The body and organ weights are given in Table VI.

TABLE IV.

Age.	Male.			Female.		
	Thyroid 1:5,000.	Thyroid 1:20,000.	Control.	Thyroid 1:5,000.	Thyroid 1:20,000.	Control.
days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
55	85	81	82	78	74	77
58	92	89	88	85	80	83
61	98.5	95	95	86	85.5	91
64	103	103	103	88	90	97
67	104	103	106	86	94	97
70	105	108	114	88	100	106
73	108	114	122	88	105	115
76	107	119	128	87	107	117
79		119.5	133	84	106	116
82		126	136		106	122
85		126	136		106	130
88		132	146		112	128
91		135	152		114	131
94		129	160		119	135
Weight increase.						
In 18 days.....	15	30	40	2	27	34
" 36 "		40	72		39	52

Experiment 6.—Comparison of different thyroid preparations. The litter, born on February 7th, 1920, consisted of six males. Two were used for iodide tests. The remainder were treated as in Experiment 5. The control was fed bread and milk only. Treatment was commenced on the 56th day. Dose 1: 5,000. The body and organ weights are given in Table VII.

No. 1 developed marked and unmistakable tetany symptoms on the 71st day (15th day of treatment). The spasms were accompanied by typical flexing of the fore limbs and paws and dragging of the hind limbs. Similar attacks were noticed during the 72nd, 74th, 75th, and 78th days, and others were suspected to have occurred at night, from the condition of the animal in the morning. During this period the rat exhibited a con-

TABLE V.

	Male. Thyroid; 1:5,000.	Control figures from Donaldson's tables for males.			Female. Thyroid; 1:5,000.	Control figures from Donaldson's tables for females.		
		Same age.	Same length.	Same weight.		Same age.	Same length.	Same weight.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Body weight	107	121.1	83.9	106.7	79	121.3	84.4	79
Liver	8.05	6.94	5.31	6.33	5.14	6.92	5.33	5.11
Kidneys	2.25	1.112	0.824	1.002	1.78	1.108	0.828	0.791
Heart	0.85	0.545	0.414	0.496	0.78	0.544	0.416	0.399
Lungs	1.17	0.778	0.585	0.704	1.12	0.776	0.587	0.562
Spleen	0.31	0.334	0.239	0.298	0.308	0.333	0.240	0.228
Adrenals	0.043	0.0250	0.0200	0.0231	0.054	0.0338	0.0249	0.0237
Thyroid	0.0097	0.0220	0.0166	0.0200	0.0104	0.0219	0.0167	0.0160
Body length, *mm*	153	173	153	166	150	169	150	147

	Male.		Female.		Male.		Female.	
	Thyroid; 1:20,000.	Control.	Thyroid; 1:20,000.	Control.	Thyroid.	Control.	Thyroid.	Control.
	gm.	*gm.*	*gm.*	*gm.*	*per cent**	*per cent**	*per cent**	*per cent**
Body weight	129	160	119	135				
Liver	9.91	7.12	8.41	7.12	7.68	4.45	7.07	5.27
Kidneys	1.82	1.56	1.90	1.37	1.40	0.97	1.67	1.37
Heart	0.85	0.79	0.78	0.75	0.66	0.50	0.65	0.55
Lungs	0.85	0.92	1.01	0.71	0.66	0.57	0.84	0.53
Spleen	0.398	0.419	0.447	0.423	0.31	0.26	0.37	0.31
Thyroid, fresh	0.0084	0.0127	0.0096	0.0108	0.0065	0.0079	0.0080	0.0080
" dry	(0.0026)	(0.0037)	(0.0033)	(0.0033)	(0.0020)	(0.0023)	(0.0028)	(0.0025)
Body length, *mm*	170	182	160	169				

* Computed on body weight.

TABLE VI.

Age.	Rat 1. Thyroid A; 0.38% I.	Rat 2. Thyroid B; 0.34% I.	Rat 3. Thyroid C; 0.18% I.	Rat 4. Liver. Control.	Rat 5. Control.
days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
40	73	74	68	66	60
43	76	81	73	70	65
46	79	82	76	71	66
49	81	85	84	82	75
52	84	89	88.5	88	83
55	89	92	90.5	95	86
58	91.5	94.5	94.5	98	92
61	95	100.5	99.5	105.5	101.5
63	95	101	103.5	107	106
Weight increase.					
In 17 days	16	19	27.5	36	40
Body length, *mm.*	170	170	180	175	175
Weight of organs.					
Liver	8.79	7.90	6.99	5.71	4.99
Kidneys	1.58	1.39	1.40	1.10	1.13
Heart	0.72	0.66	0.60	0.52	0.61
Lungs	0.64	0.73	0.58	0.63	0.68
Testes	1.86	1.93	1.96	2.06	1.88
Spleen	0.323	0.316	0.328	0.250	0.281
Adrenals	0.025	0.025	0.021	0.018	0.017
Thyroid, fresh	0.0084	0.0081	0.0077	0.0138	0.0100
" dry	(0.0021)	(0.0015)	(0.0018)	(0.0035)	(0.0027)
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Liver	9.25	7.82	6.75	5.33	4.71
Kidneys	1.66	1.38	1.35	1.04	1.07
Heart	0.76	0.65	0.58	0.49	0.57
Lungs	0.68	0.73	0.56	0.59	0.59
Testes	1.96	1.92	1.89	1.93	1.77
Spleen	0.34	0.31	0.32	0.23	0.26
Adrenals	0.026	0.025	0.020	0.017	0.016
Thyroid, fresh	0.0089	0.0081	0.0074	0.0129	0.0094
" dry	(0.0022)	(0.0015)	(0.0017)	(0.0033)	(0.0025)

tinuous very rapid heart beat, rapid respiration, was restless, and developed a rough coat. There was no exophthalmos. There was a gradual protusion in front and above the thyroid cartilage, external to the muscular coat; pus developed and a scab formed. The tissue surrounding this was examined histologically, and appeared to be abnormal lymphatic tissue.

TABLE VII.

Age.	Rat 1. Thyroid A; 0.38% I.	Rat 2. Thyroid B; 0.34% I.	Rat 3. Thyroid C; 0.18% I.	Rat 4. Control.
days	*gm.*	*gm.*	*gm.*	*gm.*
50	50	50	46	50
53	58.5	56	50.5	56
56	61.5	61	58.5	63
59	65.5	63	64	72.5
62	68.5	66	67	80
65	75	71	76	88
68	78	75	81.5	96
71	79	77	83	102
74	76	85.5	92.5	111
77	79	90	98	122
80	76	90	103	129
82	75	93	109	134.5
Weight increase.				
In 18 days	14.5	24.5	34	48
" 26 "	13.5	32	50.5	71.5
Body length, *mm*	146	165	165	185
Weight of organs.				
Liver	7.72	7.64	8.05	8.01
Kidneys	2.05	1.39	1.41	1.26
Heart	0.61	0.72	0.68	0.60
Lungs	0.60	0.65	0.73	0.80
Testes	1.67	1.89	2.08	2.17
Spleen	0.230	0.402	0.398	0.330
Adrenals	0.048	0.025	0.022	0.019
Thyroid, fresh	0.0058	0.0074	0.0085	0.0125
" dry	(0.0015)	(0.0021)	(0.0028)	(0.0035)
	per cent	*per cent*	*per cent*	*per cent*
Liver	10.29	8.23	7.39	5.94
Kidneys	2.73	1.49	1.29	0.94
Heart	0.81	0.77	0.62	0.45
Lungs	0.80	0.70	0.67	0.59
Testes	2.23	2.03	1.91	1.61
Spleen	0.31	0.43	0.37	0.25
Adrenals	0.064	0.027	0.020	0.014
Thyroid, fresh	0.0077	0.0080	0.0078	0.0093
" dry	(0.0020)	(0.0022)	(0.0025)	(0.0026)

The muscular development was small, compared with the normal animal of the group. No. 2 had a rapid heart beat, was restless, developed a rough coat, and a doubtful exophthalmos. Towards the end of the experiment a marked protuberance appeared in a position similar to that in the first rat (a pus tumor). The appearance and condition of the other two rats were normal until they were killed.

Experiment 7.—Comparison of different thyroid preparations. An experiment with a litter of four female rats, born March 15th, 1920. The same treatment was given as in the two previous experiments. Thyroid feeding was commenced on the 39th day. Dose 1:5,000. The body and organ weights are given in Tables VIII and IX.

TABLE VIII.

Age.	Rat 1. Thyroid A; 0.38% I.	Rat 2. Thyroid B; 0.34% I.	Rat 3. Thyroid C; 0.18% I.	Rat 4. Control.
days	*gm.*	*gm.*	*gm.*	*gm.*
39	47	40	43.5	36
42	49	42.5	48	42
45	55.5	46	54.5	49
48	62	53	59	54.5
51	67.5	61	66	63.5
54	74	65	73	70
57	85	73	80	79.5
Weight increase.				
In 18 days.....	38 (81%)	33 (82%)	36.5 (84%)	43.5 (121%)
Body length, *mm*.	156	148	155	150

Experiment 8.—An endeavor to induce tetany by thyroid feeding. Ten young rats from different litters were fed thyroid over a long period. An unlimited bread and milk diet was given. The initial ages were not known. Feeding commenced May 8th, 1920. The first five were fed at the rate of 1:2,000; the second five at 1:5,000.

No. 1, female, initial weight 51 gm. On the 8th day of feeding thyroid, the rat showed symptoms of incipient tetany, on the ninth day appeared normal, and on the 10th day had an attack of tetany lasting 3 hours, with rapid pulse and respiration, high temperature, and fatal termination. The weight at death was 70 gm. The animal was autopsied. The liver presented a peculiar mottled appearance, the heart was empty (hard), and the blood was chiefly in the periphery. The other organs appeared normal.

No. 2, male, initial weight 57 gm. On the 18th day of treatment the animal appeared abnormal. The coat was ragged. There was no distinct symptom of tetany. Death occurred on the 19th day. For several days prior to death there was a permanent stricture of the penis. Weight at death 67 gm.

TABLE IX.

	Rat 1.	Rat 2.	Rat 3.	Rat 4.	Rat 1.	Rat 2.	Rat 3.	Rat 4.
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*
Liver	5.70	5.50	5.15	4.02	6.71	7.53	6.44	5.03
Kidneys	1.17	1.04	1.13	0.75	1.38	1.42	1.41	0.94
Heart	0.65	0.55	0.55	0.49	0.76	0.75	0.69	0.61
Lungs	0.78	0.60	0.71	0.62	0.94	0.82	0.89	0.78
Spleen	0.462	0.395	0.407	0.308	0.54	0.54	0.51	0.39
Adrenals	0.026	0.023	0.026	0.019	0.030	0.031	0.032	0.024
Thyroid, fresh	0.0060	0.0074	0.0065	0.0062	0.0070	0.0101	0.0080	0.0078
" dry	0.0020	0.0027	0.0022	0.0017	0.0024	0.0037	0.0027	0.0021

TABLE X.

	Rat 5. Thyroid A; 1:2,000.	Control figures from Donaldson.		Rat 9. Thyroid A; 1:5,000.	Control figures from Donaldson.		Rat 10. Thyroid A; 1:5,000.	Control figures from Donaldson.	
		Same length.	Same weight.		Same length.	Same weight.		Same length.	Same weight.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Body weight	133	181.2	**132.3**	160	169.1	**160.5**	112	157.7	**112.6**
Body length, *mm.*	196	**196**	178	192	**192**	189	188	**188**	169
Liver	11.47	9.33	7.40	13.87	8.86	8.53	11.60	8.42	6.58
Kidneys	2.05	1.568	1.198	2.35	1.477	1.412	2.15	1.391	1.047
Heart	0.97	0.742	0.584	1.19	0.704	0.676	0.89	0.667	0.517
Lungs	1.15	1.083	0.836	1.31	1.022	0.979	1.14	0.965	0.735
Testes	1.91	2.051	1.654	2.16	1.964	1.898	2.35	1.876	1.450
Spleen	0.535	0.487	0.363	0.699	0.456	0.435	0.371	0.428	0.313
Adrenals	0.062	0.0321	0.0264	0.035	0.0307	0.0297	0.038	0.0294	0.0239
Thyroid	0.0139	0.0299	0.0235	0.0168	0.0284	0.0272	0.0099	0.0269	0.0208

No. 3, male, initial weight 134 gm. Died on the 27th day of treatment. Final weight 96 gm. The weight fell steadily throughout the treatment. There was occasional stricture of the penis after the 17th day. No distinct cause of death was noted.

No. 4, male, initial weight 130 gm. Death occurred on the 30th day, from no distinct cause. There was a steady slow fall in weight, the final weight being 112 gm.

No. 5, male, initial weight 115 gm. There was a slow gain in weight. On the 32nd day the animal (weight 133 gm.) was killed and examined (see Table X).

No. 6, male, initial weight 78 gm. The weight kept practically constant (maximum noted was 84 gm.). The animal died on the 17th day (weight 78 gm.). There were no symptoms of tetany and no distinct cause of death.

No. 7, male, initial weight 97 gm. The weight varied between 93 and 100 gm. for 16 days, and then fell slowly. The animal died on the 28th day from no observed cause. Final weight 88 gm. Occasional stricture of the penis was noted.

No. 8, male, initial weight 89.5 gm. Weight kept fairly constant (maximum reached, 97 gm.). Death occurred on the 29th day from no definite cause. Weight 84 gm.

No. 9, male, initial weight 115.5 gm. There was a slow increase in weight to 160 gm. on the 32nd day when the animal was killed and examined (see Table X). The appearance remained normal throughout, except that bald patches developed around the eyes.

No. 10, male, initial weight 115.5 gm. This increased to 128 gm. (11th day) and then fell slowly to 112 gm. (32nd day) when the animal was killed and examined (see Table X). The hair was somewhat rough.

It should be remarked further that the examination of No. 5 showed the development of numerous small lymphoid glands, especially in the neighborhood of the axilla. Several of the animals showed a marked inflammation (granulation) of the eyelids.

Experiment 9.—Sodium iodide. Two male rats from the same litter, born September 8th, 1919. The iodide dosage was made equivalent to the amount of iodine in the Thyroid A preparation fed in the ratio of 1:5,000 (1 part of iodine to 1,250,000 of body weight). The initial weights of the iodide and control rats were respectively 102 and 99 gm. (66 days old); the weights after feeding 36 days were 171 and 169 gm. At no time was the difference in weight greater than 7 gm. and it varied in both directions.

The weights of the following organs were respectively for the iodide and control rats: liver, 8.03 and 6.91 gm.; kidneys, 1.45 and 1.28 gm.; heart, 0.76 and 0.72 gm.; lungs, 0.92 and 0.83 gm.; spleen, 0.53 and 0.45 gm.; thyroid, fresh, 0.0122 and 0.0125 gm.; dry, calculated from one lobe, 0.0037 and 0.0043 gm.

Experiment 10.—Sodium iodide. One of two female rats from the litter born January 5th, 1920, was given sodium iodide in the ratio of 1 part of

iodine to 500,000 of body weight. The iodide was fed from the 46th day. Compare Experiment 5. The results are given in Tables XI and XII.

TABLE XI.

Age.	Iodide rat.	Control rat.
days	*gm.*	*gm.*
40	61	60
43	65	63
46	67	61
49	77	68
52	80.5	70
55	85.5	73
58	90	74
61	94.5	79
63	100	82
Body length, *mm*.....	165	160

TABLE XII.

	Iodide rat.	Control rat.	Iodide rat.	Control rat.
	gm.	*gm.*	*per cent*	*per cent*
Liver.....	5.01	3.88	5.01	4.73
Kidneys.....	1.12	0.89	1.12	1.08
Heart.....	0.60	0.45	0.60	0.55
Lungs...	0.65	0.55	0.65	0.67
Spleen.....	0.254	0.227	0.25	0.28
Adrenals.....	0.023	0.019	0.023	0.023
Thyroid, fresh.....	0.0090	0.0092	0.0090	0.0112
" dry.....	(0.0019)	(0.0018)	(0.0019)	(0.0022)

The control rat during this period did not appear to be perfectly normal, and did not take food well.

Experiment 11.—Sodium iodide. Two of the six rats born February 7th, 1920 (see Experiment 6), were fed different doses of iodide, and the same control was used as for those fed thyroid. Feeding was commenced on the 56th day. The results are given in Tables XIII and XIV.

Experiment 12.—Sodium iodide. A litter of eight rats, males, born April 6th, 1920. Two rats (Nos. 1 and 2) were used as controls, two were fed iodide at the rate of 1 part of iodine to 50,000 of body weight (Nos. 3 and 4), two at the rate of 1 of iodine to 25,000 (Nos. 5 and 6), and two at the rate of 1 of iodine to 12,500 (Nos. 7 and 8). Feeding was commenced on the 41st day. The results are given in Table XV.

The thyroid material from each pair of rats was united and the iodine estimated by Kendall's (1920) method. The results are given in Table XVI.

TABLE XIII.

Age.	Rat 1. Iodine 1.5:500,000.	Rat 2. Iodine 3:500,000.	Control rat.
days	*gm.*	*gm.*	*gm.*
50	54.5	51.5	50
53	59.5	56.5	56
56	68	62	63
59	75	71	72.5
62	83	74.5	80
65	92	85	88
68	98	93	96
71	105	97	92
74	114	105	111
77	120	113.5	122
80	126.5	117.5	129
82	131	123	134.5
Weight increase.			
In 18 days	46	43	58
" 26 "	63	61	71.5
Body length, *mm*	170	180	185

TABLE XIV.

	Rat 1.	Rat 2.	Control rat.	Rat 1.	Rat 2.	Control rat.
	gm.	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Liver	6.44	6.54	8.01	4.92	5.32	5.94
Kidneys	1.19	1.10	1.26	0.91	0.89	0.94
Heart	0.54	0.58	0.60	0.41	0.47	0.45
Lungs	0.81	0.68	0.80	0.62	0.55	0.59
Spleen	0.299	0.308	0.330	0.23	0.25	0.25
Testes	2.27	2.22	2.17	1.73	1.80	1.61
Adrenals	0.027	0.024	0.019	0.021	0.020	0.014
Thyroid, fresh	0.0129	0.0140	0.0125	0.0098	0.0114	0.0093
" dry	(0.0043)	(0.0042)	(0.0035)	(0.0033)	(0.0034)	(0.0026)

Experiment 13.—Rabbits; Thyroid A; 1:5,000. Litter born March 29th, 1920. Five survived; three males, and two females. Thyroid feeding commenced on the 50th day. The thyroid was mixed with oatmeal. The control female appeared abnormal on the 83rd day, and died on the 87th day. There was apparent paralysis of the hind limbs. Examination after death showed an abnormally distended bladder, very distended stomach, and an apparently abnormal spleen. The other animals appeared normal. They were killed on the 87th day. The results are given in Tables XVII and XVIII.

TABLE XV.

Age.	Rat 1; control.	Rat 2; control.	Rat 3; 1:50,000.	Rat 4; 1:50,000.	Rat 5; 1:25,000.	Rat 6; 1:25,000.	Rat 7; 1:12,500.	Rat 8; 1:12,500.
days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
41	41	43	45	50	45	41	45	40
44	50	49	53	57	55	48	50	43
47	53	55	59	60	62	56	58	49
50	59	63	64	70	67	58	62	53
53	64	70	70	79	74	63	68	63
56	69	75	77	86	83	69	74	70
59	78	80	86	95	94	74	79	75
62	85	86	98	106	102	82	86	85
65	93	93	105	118	118	85	91	90
68	100	106	115	125	124	93	98	99
71	104	110	118	132	133	100	105	102
74	116	121	131	143	144	106	112	110
77	121	128	137	152	150	112	119	119
80	132	139	145	163	161	115	128	131
Weight increase.								
In 18 days	37	37	41	45	49	33	34	35
" 36 "	80	85	92	102	105	71	74	79
" 39 "	91	96	100	113	116	74	83	91
Body length, *mm*	190	184	184	185	196	180	185	183

Weight of organs.

Liver	7.73	7.66	8.11	7.79	7.70	5.55	6.72	5.98
Kidneys	1.25	1.28	1.17	1.31	1.26	0.97	1.06	0.97
Heart	0.54	0.64	0.62	0.70	0.66	0.48	0.56	0.54
Lungs	0.86	0.85	1.13	1.11	0.96	0.87	0.91	0.95
Testes	1.92	2.13	2.14	2.25	2.24	1.98	2.09	1.98
Spleen	0.427	0.426	0.454	0.524	0.446	0.325	0.367	0.404
Adrenals	0.023	0.023	0.023	0.023	0.022	0.021	0.020	0.017
Thyroid, fresh	0.0151	0.0123	0.0125	0.0145	0.0155	0.0117	0.0112	0.0111
" dry	0.0058	0.0036	0.0038	0.0042	0.0045	0.0034	0.0032	0.0032
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Liver	5.85	5.51	5.59	4.78	4.78	4.83	5.25	4.56
Kidneys	0.95	0.92	0.81	0.80	0.78	0.84	0.83	0.74
Heart	0.41	0.46	0.43	0.43	0.41	0.42	0.44	0.41
Lungs	0.65	0.61	0.78	0.68	0.60	0.76	0.71	0.72
Testes	1.45	1.53	1.48	1.38	1.39	1.72	1.63	1.51
Spleen	0.32	0.31	0.31	0.32	0.28	0.28	0.29	0.31
Adrenals	0.017	0.016	0.016	0.014	0.014	0.018	0.016	0.013
Thyroid, fresh	0.0114	0.0088	0.0086	0.0089	0.0096	0.0102	0.0087	0.0085
" dry	0.0029	0.0026	0.0026	0.0026	0.0028	0.0030	0.0025	0.0024

TABLE XVI.

Material.	Amount taken.	Iodine found.	
	gm.	gm.	per cent
Thyroids from Rats 1 and 2.....	0.0074	0.0000061	0.08
" " " 3 " 4.....	0.0080	0.0000258	0.32
" " " 5 " 6.....	0.0079	0.0000214	0.27
" " " 7 " 8.....	0.0064	0.0000170	0.27

Histological Examination.

Where in the tables figures for dried thyroid are given in parentheses, one lobe was transferred fresh to corrosive sublimate, sectioned, and examined histologically. Professor Swale Vincent kindly reported on these sections. He found that in every case not only had colloid distended the vesicles, but that the lymphatic vessels outside the vesicles, and the intervesicular tissue were also distended with colloid; that, in fact, the colloid had overflowed. Small doses over long periods produced effects comparable with larger doses over a short period, and the effects of administration of sodium iodide were the same as those of administration of thyroid.

In Experiment 5, liver, kidney, heart, lung, testes, spleen, and adrenal sections were made from No. 2, fed thyroid containing 0.34 per cent of iodine. These showed no observable difference from sections from the corresponding organs of the control rat.

DISCUSSION.

Results for Normal Rats.

The gross weights and body lengths show in individual cases marked differences from Donaldson's figures, though the average results agree moderately well. The organ weights, where measured, show distinct deviations from Donaldson's tables, as shown in Table XIX.

The differences for the spleen, which indicate variations both above and below the averages recorded for animals of the same age, weight, and body length, can be taken as typical also of the figures for liver, heart, kidneys, and lungs. In the case of the testes our figures appear to be distinctly higher than Donaldson's.

TABLE XVII.

Age.	Rabbit 1; male. Thyroid.	Rabbit 2; male. Thyroid.	Rabbit 3; male. Control.	Rabbit 4; female. Thyroid.	Rabbit 5; female. Control.
days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
38	297	324	335	313	267
44	307	363	345	330	298
50	330	379	351	339	309
56	335	374	420	320	383
62	355	395	463	260	409
68	425	472	535	344	469
74	455	498	559	379	482
80	488	511	603	390	517
86	554	561	639	410	524
87	543	567	658	415	(490)
Weight increase.					
In 18 days	95	93	184	5	160
" 36 "	224	182	288	71	215
Body length, *mm*	300	315	330	325	280
Weight of organs.					
Liver	29.29	25.94	26.57	25.94	21.40
Kidneys	5.44	5.44	5.56	4.46	5.15
Heart	2.35	2.67	1.66	2.35	2.35
Lungs	3.86	3.93	3.92	2.67	2.35
Testes	0.082	0.061	0.168		
Ovaries				0.030	0.033
Spleen	0.301	0.294	0.298	0.239	0.102
Adrenals	0.174	0.134	0.189	0.124	0.190
Pancreas	1.071	0.907	1.078	0.743	0.582
Thyroid, fresh	0.0313	0.0450	0.0456	0.0304	0.0325
" dry	0.0133	0.0203	0.0174	0.0124	0.0130
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Liver	5.39	4.58	4.04	6.25	4.04
Kidneys	1.00	0.96	0.84	1.07	0.97
Heart	0.43	0.47	0.25	0.56	0.44
Lungs	0.71	0.71	0.60	0.64	0.44
Testes	0.015	0.011	0.025		
Ovaries				0.0072	0.0062
Spleen	0.055	0.052	0.045	0.058	0.019
Adrenals	0.032	0.024	0.029	0.030	0.036
Pancreas	0.20	0.16	0.16	0.18	0.11
Thyroid, fresh	0.0058	0.0079	0.0069	0.0073	0.0061
" dry	0.0025	0.0036	0.0026	0.0030	0.0025

The discrepancy becomes more marked with the adrenals,[3] all of lower weight, and still more marked with the thyroids, the weights of which are only from one-third to one-half those of Donaldson's rats. These discrepancies may to some extent be due to differing diets. In regard to the adrenals it is now definitely proved (McCarrison (1919), Vincent and Hollenberg (1920)) that inanition causes marked hypertrophy. Such hypertrophy is also very distinct with thyroid feeding, and it is possible that other diet modifications may also cause changes. The thyroid discrepancies are more difficult to explain. Hoskins and Herring also obtained lower normal figures.

TABLE XVIII.

Estimation of Iodine Content of Thyroids.

Material.	Amount taken.	Iodine found.	
	gm.	*gm.*	*per cent*
Thyroids of Rabbit 1	0.0133	0.0000293	0.22
" " " 2	0.0203	0.0000642	0.32
" " " 3	0.0174	0.0000682	0.39
" " " 4	0.0124	0.0000350	0.29
" " " 5	0.0130	0.0000476	0.37

No clue is afforded from these figures as to whether comparisons should be made more appropriately with rats of the same age, weight, or body length. It is quite evident that wherever possible control rats of the same age from the same litter must always be used. Even with such animals distinct variations are evident, and it is also obvious that where there is a marked slowing of growth comparison of the actual weights of organs may lead to an incorrect conclusion. We consider that the comparisons least likely to lead to error are those of figures expressing percentage of organ weight to total body weight for animals of the same age although there will be with these an

[3] The adrenal figures are not included in the table. The following, for the control rat from Experiment 12, can be taken as typical: weight 0.023 gm.; Donaldson's figures, same age 0.0264 gm., same weight 0.0264 gm., same length 0.0300 gm.

TABLE XIX.

Experiment No.	Rat.	Weight.	Age.	Length.	Spleen.	Donaldson's figures for spleen.			Testes.	Donaldson's figures for testes.			Thyroid.	Donaldson's figures for thyroid.		
						Same age.	Same weight.	Same length.		Same age.	Same weight.	Same length.		Same age.	Same weight.	Same length.
		gm.	*days*	*mm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
5	Control male.	106	63	175	0.281	0.247	0.298	0.345	1.88	1.125	1.382	1.586	0.0100	0.0171	0.0200	0.0226
12	" "	132	80	190	0.427	0.363	0.363	0.442	1.92	1.654	1.654	1.920	0.0151	0.0235	0.0235	0.0276
6	" "	134.5	82	185	0.330	0.375	0.369	0.407	2.17	1.698	1.675	1.809	0.0125	0.0242	0.0238	0.0258
12	" "	139	80	184	0.426	0.363	0.363	0.400	2.13	1.721	1.721	1.787	0.0123	0.0245	0.0245	0.0255
4	" "	152	91	182	0.419	0.414	0.414	0.388					0.0084	0.0262	0.0262	0.0248
7	" female.	79.5	57	150	0.308	0.208	0.228	0.240					0.0062	0.0148	0.0160	0.0167
10	" "	82	63	160	0.227	0.236	0.236	0.285					0.0092	0.0164	0.0164	0.0193
4	" "	131	91	169	0.423	0.375	0.356	0.333					0.0108	0.0244	0.0232	0.0219

error of a small per cent for thyroid-fed animals from fat loss (compare Experiment 2). No great stress can be laid on the comparisons with Donaldson's figures in Experiment 8 since the ages of the animals were not known.

Effect of Thyroid Feeding on Total Body Weight.

Under the conditions of experiment adopted, in each of the seven experiments on rats, and that on rabbits, a distinct and marked decrease of growth rate is shown *without exception*, when animals are fed thyroid gland. This decrease is greater than any variation between animals in the same litter. The effect is usually perceptible after 2 or 3 days feeding. With prolonged feeding of small doses (Experiments 1 and 2, females) the body weight tends to become normal again. This is probably due to the fat loss (and perhaps the total loss through the decreased growth rate) becoming balanced by the hypertrophy of certain organs (Experiment 2 indicates a fat loss of about 4 per cent of the body weight). Large doses tend to inhibit growth completely (Experiment 8).

The same decrease in growth rate occurs in rabbits.

This decrease is not to be attributed to any toxic effect of thyroid other than that specific to thyroid tissue itself, at any rate with doses not greater than one-five thousandth of the body weight, since the equivalent amount of liver tissue, dried by methods certainly not more careful to exclude autolysis etc., produces no such effect.

The decrease in growth rate is definitely greater the greater the amount of thyroid fed (Experiment 4), but in order to show this it is necessary to use animals from the same litter (compare Experiments 2 and 3).

Analysis of the results detailed above does not indicate marked difference between females and males in susceptibility to thyroid feeding (*cf.* Herring).

The decrease in growth rate is greater the greater the iodine content of the thyroid fed (Experiments 5 and 6; Experiment 7 does not show this so accurately for the gross changes, but this can almost certainly be accounted for by the greater differences in initial weight; the percentage increases in weight are not in disagreement with the conclusion).

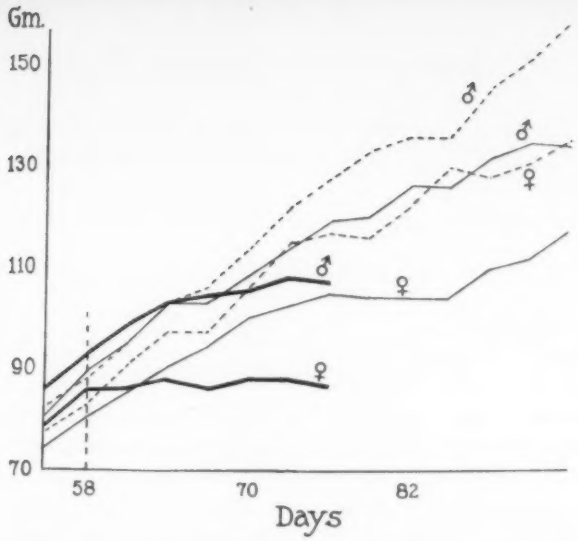

FIG. 1. Experiment 4. The heavy lines represent weights of rats dosed in the ratio of 1:5,000, the light lines those for the ratio 1:20,000, and the dotted lines those for control rats.

Thyroid feeding was commenced where indicated by the vertical dotted line.

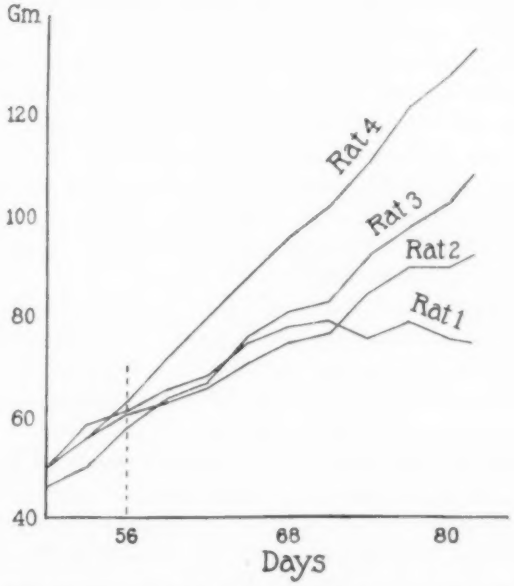

FIG. 2. Experiment 6. Rat 1, fed thyroid containing 0.38 per cent iodine; Rat 2, fed thyroid containing 0.34 per cent iodine; Rat 3, fed thyroid containing 0.18 per cent iodine; Rat 4, control rat.

Thyroid feeding was commenced where indicated by the vertical dotted line.

Fig. 1 illustrates the effect of dosage on the extent of decrease, and Fig. 2 the effect of iodine content of the thyroid fed.

Effect of Thyroid Feeding on Body Length (Snout to Anus).

There is usually, but not invariably, a decrease in body length.

Effect of Thyroid Feeding on the Liver, Kidneys, Heart, Spleen, Lungs, Testes, and Adrenals.

The livers of nine rats out of eleven (Experiments 4, 5, 6, and 7) show a distinct hypertrophy from comparison of the actual weights. Expressed as percentages, the figures emphasize the hypertrophy, and those of the remaining two rats conform. The figures (actual weight) for kidneys show an increase in every case. There is one exception for heart, and one for spleen; expressing these in percentages brings them into agreement. The adrenals invariably show an actual increase (nine rats, Experiments 5, 6, 7; no accurate observations were made in Experiment 4). The figures for testes and lungs are less in accordance, but the percentage figures in most cases indicate a relative hypertrophy. The extent of the hypertrophy is evidently less for these two organs. The somewhat greater extent of general hypertrophy obtained by Herring would appear to be due to longer feeding with larger doses. Our figures, correlated with those of Hoskins, of Herring, and of Hewitt, lead to the general conclusion that the extent of hypertrophy depends on the size of dose and the length of dosage period, although variations with individual litters are marked.

Hewitt's results are open to criticism, since apparently Donaldson's figures alone were used for comparison. Nevertheless his conclusion that these organs tend to resume normal proportions after resumption of ordinary diet is valid, since it is based on observations on rats of the same litter.

It is probable that Herring's contention that part at least of the thyroid effect is produced through the medium of the hypertrophied adrenal may be correct in as far as the testes are concerned, since Hoskins (1916) noted that adrenal feeding produced hypertrophy of these organs. Since Hoskins' results indicated no effect on other organs, it is probable that the effects on heart,

pancreas, adrenals, kidneys, and liver are due directly to thyroid action. Herring has pointed out that Hatai (1915) has induced similar hypertrophies in rats subjected to a large amount of exercise, a treatment which may logically be compared with the increase of general metabolic activity now usually regarded as the chief function of the thyroid secretion.

Rabbits showed a relative hypertrophy except for testes, ovaries, and adrenals. There was no constant decrease in thyroid. The water content of the thyroid, both in normal and treated animals, was unusually low.

The percentage hypertrophies recorded in Experiments 5 and 6 point to greater hypertrophy with greater iodine content of thyroid fed. Experiment 7 only partly confirms this. While further experiments are desirable to exclude individual variations we conclude that the results indicate that this dependency on iodine content exists. The proportionality of the action on both growth rate and organ hypertrophy to iodine content, in harmony with other observations on the physiological and metabolic action of thyroid preparations, is to be expected if thyroxin is the essential constituent of the thyroid, since, according to Kendall (1915), this compound occurs combined in the gland in amount invariably corresponding to one-fourth of the total iodine content.

Effect of Thyroid Feeding on Size of Thyroid.

Our results confirm Herring's, that in growing young rats fed thyroid the thyroid shows relatively less growth. We obtained a greater effect with males than with females. The thyroids as a rule were paler than normal glands. Their histological appearance has been described. The figures for dry thyroid tissue also show a similar decrease.

Effect of Thyroid Feeding on Body Fat and Muscular Development.

Like most of the previous observers we have noted an almost complete disappearance of fat tissue in all animals fed thyroid. In one or two cases, *e.g.* No. 1, Experiment 6, the muscular development was distinctly less than normal.

Effect of Feeding Sodium Iodide.

Examination of the figures in Experiments 9 to 12 reveals no decrease in growth rate, no relative hypertrophy of body organs, and no effect on weight of thyroid. No disappearance of fat tissue was observed. The actual figures probably indicate fairly accurately the extent of variation in normal rats of the same litter. It has already been noted that histological examination of the thyroid shows the same changes after iodide as after thyroid feeding.

Other Effects from Thyroid Feeding.

The occasional deaths noted during thyroid feeding by previous observers, and occurring in our experiments especially with the larger doses, are presumably to be regarded as a definite result of thyroid action. Kendall (1919) has noted the same effect with thyroxin. The non-occurrence of such results when thyroid medication is given in diseases such as myxodema and cretinism over prolonged periods is explicable, since the thyroid deficiency is merely corrected, and the effect of a hypersecretion is not produced. Other hypersecretion effects (rough coat, increased heart beat, etc.) have been observed by us in but few cases, and then chiefly with young animals. These seem distinctly more susceptible to the same relative dose. The two cases of tetany that we observed were with young animals. While we have no explanation to offer for this occurrence, we would point out that the observations of Collip and Backus (1920), and of Grant and Goldman (1920), that prolonged forced breathing may cause symptoms of tetany in man, may have some bearing, since rapid respiration and heart beat were concomitant factors in the cases we observed.

Effect of Thyroid and of Iodide Feeding on the Iodine Content of the Thyroid.

Experiment 12 shows that there is a marked increase in iodine content after administration of iodide, in agreement with numerous previous observations. The amount of iodide fed, within the limits of this experiment, did not affect the amount of increase.

No such increase was observed in rabbit thyroids after thyroid feeding. This suggests that with the relatively small dose employed no iodine was stored in the thyroid, but that the iodine-containing compounds in the thyroid fed were entirely used up in producing the systemic effects, and the iodine was subsequently excreted.

Effect of Thyroid Preparations of Different Age.

The very similar effects produced by the 9 year old Merck, and the recently prepared hog thyroid preparation (Armour) indicate that age has no deteriorating effect on carefully prepared desiccated thyroid. Hoskins has already shown that desiccated and fresh glands produce the same results, in confirmation of the observations of previous workers on other thyroid effects.

SUMMARY.

The chief results described in this paper are:

1. Continued small doses of desiccated thyroid gland fed to young white rats produce (*a*) a definite and invariable decrease in growth rate; (*b*) hypertrophy of the organs concerned with increased metabolism—heart, liver, kidneys, adrenals, etc. (confirmatory of Hoskins and Herring); (*c*) disappearance of fat (confirmatory of Hoskins and Herring).

2. The decrease in rate of growth is proportional to (*a*) the amount of thyroid fed and (*b*) the iodine content of the thyroid fed.

3. The hypertrophy varies with dose and length of application of dose, and appears to be proportional to the iodine content of the dose.

4. Sodium iodide fed in quantities varying from amounts equal in iodine content to the thyroid doses to amounts 100 times as great produces no effect on growth rate, and no hypertrophy.

5. The effects produced are not due to protein feeding, autolysis products, or any similar cause, but specifically to thyroid tissue, or some constituent of it.

6. Both thyroid and iodide feeding increase the colloid in the thyroid (confirmatory of Fordyce, and of Kojima). Thyroid feeding inhibits the growth rate of the thyroid (confirmatory of Herring). Iodide does not produce this effect.

7. Similar effects from thyroid feeding on growth rate and organ hypertrophy were obtained with rabbits.

Since of numerous tissues tested only thyroid produces a definite effect (Hoskins) and since iodide does not produce this effect, we suggest that decreased growth rate, organ hypertrophy (especially of the liver, heart, kidneys, and adrenals), and relatively decreased thyroid can be used as discriminatory tests for preparations alleged to be the essential thyroid secretion.

We desire to acknowledge our indebtedness to Professor Swale Vincent for the interest that he has taken in this work, and for the valuable suggestions that he has made to us in the course of it.

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RELATIONSHIP BETWEEN THE PLASMA BICARBONATE AND URINARY ACIDITY FOLLOWING THE ADMINISTRATION OF SODIUM BICARBONATE.

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Palmer and Van Slyke (1) have shown that the urine of normal men approaches the alkalinity of the blood (pH = 7.4) when the plasma bicarbonate CO_2 exceeds 71 ± 5 volumes per cent, while the urines of many pathological cases, particularly cases with a high grade of renal disease and diabetes mellitus, do not approach the alkalinity of the blood until a higher plasma bicarbonate than in normal individuals has been reached. In certain instances the level of blood bicarbonate was well within the range where tetany is known to occur. Attention was called to the danger of using the urine reaction, pH = 7.4, as a safe criterion in the therapeutic use of sodium bicarbonate, but preferably in view of the evidence then available, to control alkali therapy by frequent determinations of the plasma bicarbonate. It was suggested that a more acid reaction of the urine than pH = 7.4, for instance a pH of 7.0, might be employed with advantage to control clinically the use of sodium bicarbonate in pathological cases. Furthermore, it was shown that sodium bicarbonate administered by mouth is readily absorbed and distributed in approximate uniformity to the body fluids in general as well as to the blood. The effect of a given amount of sodium bicarbonate, g, on the volume per cent of CO_2 of plasma bicarbonate, b, may be estimated from the formula, $b = \frac{38g}{W}$,

when W is the weight of the individual in kilos.[1] Estimation in this manner of the amount of sodium bicarbonate necessary to replenish the depleted alkali reserve as shown by plasma bicarbonate CO_2 determinations serve a useful purpose in clinical practice.

In view of the importance of the use of sodium bicarbonate in combating severe grades of acidosis, more especially in diabetes mellitus, nutritional diseases of children, and methyl alcohol poisoning, further observations have been made in the hope of extending the clinical applicability and reliability of the urinary reaction in controlling the administration of alkali. Although the reaction pH = 7.4 of the urine elaborated by kidneys, whose function of regulating the acid-base equilibrium of the body is impaired, is an insufficient, indeed, may be an unsafe indication that the proper amount of alkali has been given to restore the level of plasma bicarbonate to within normal limits, it was thought possible that the first significant effect of alkali administration on the urinary reaction (pH) might be employed as a safe clinical guide. That our expectations in this respect were justified is apparent from the following results.

EXPERIMENTAL.

Sodium bicarbonate in 2 gm. amounts in 100 cc. of water was given by mouth every ½ hour to subjects in whom the initial plasma bicarbonate revealed no severe grade of acidosis; that is, not less than 50 volumes per cent. In cases with a moderately severe acidosis (a plasma bicarbonate between 40 and 50 volumes

[1] The calculation in terms of plasma CO_2 is made as follows: 1 gm. of $NaHCO_3$ contains 267 cc. of CO_2 measured at 0°, 760 mm. If the body fluids are estimated at 700 cc. for each kilo of body weight, then the distribution of 1 gm. of bicarbonate among them would raise the CO_2 content in cc. per 100 cc. of fluid by $\frac{267}{7W} = \frac{38}{W}$ cc., W representing the body weight in kilos. If g gm. of sodium bicarbonate were taken into the fluids, the rise in volume per cent of CO_2 would be $\frac{38g}{W}$. Conversely, the amount of bicarbonate necessary to raise the CO_2 by b volume per cent would be $g = \frac{bW}{38}$. Hence the formula $b = \frac{38g}{W}$.

per cent), the individual doses of sodium bicarbonate were increased to 5 gm. each and when a more marked reduction of the alkali reserve (below 40 volumes per cent) was found, the dose was increased to 10 gm. It became desirable, especially in cases with severe acidosis, to give the bicarbonate every hour instead of every ½ hour. The hydrogen ion concentration of the urine was estimated immediately before the administration of each dose of alkali. Blood bicarbonate determinations were made before the sodium bicarbonate was given, and again at the point when the first significant depression of the urinary acidity was observed. It has been shown repeatedly in cases where a distinct acidosis exists and without alkali administration that, during the short interval of time of the experiments here reported, the reaction of the urine remains quite stationary. For this reason, "the first significant depression of the urinary acidity" was considered as a change corresponding to one interval in the scale of standard solutions used for the hydrogen ion concentration determinations. The standard solutions employed represented a pH of 7.4, 7.0, 6.9, 6.3, 6.0, 5.7, 5.3, 5.0, and 4.7, respectively.

The pH of the urine was determined by the colorimetric method described by Palmer and Henderson (2) with the exception that for pH values between 7.4 and 6.3 phenolsulfonephthalein was used; between 6.3 and 4.7 methyl red. A further slight modification was employed. Instead of introducing 10 cc. of urine into Florence flasks, 1 cc. of urine was pipetted into test-tubes (25 × 200 mm.) of clear glass and diluted to 25 cc. with distilled water. Under these conditions 10 drops of 0.04 per cent phenolsulphonephthalein and 10 drops of a saturated solution of methyl red produce a satisfactory color for purposes of comparison. Plasma bicarbonate estimations were made by the method described by Van Slyke and Cullen (3).

We have arranged our results in Table I in order of the grams per kilo necessary to produce a significant change in the pH of the urine. In Experiments 7, 19, and 27, before any significant change in the pH of the urine took place, the patients became nauseated, hence the experiments were discontinued.

TABLE I.

Normal and Pathological Individuals.

Experiment No.	Hospital No.	Weight.	Blood pressure.	Blood urea.	Phenolsulfonephthalein excretion in 2 hrs.	Reaction of urine.		Combined CO_2 in plasma.		Amount of $NaHCO_3$ given.		Time between taking of first and second blood samples.	Calculated increase in CO_2, $\frac{38y}{W}$.	Observed increase in CO_2.	Difference between calculated and observed CO_2 increase.	Remarks.
						Before $NaHCO_3$.	After $NaHCO_3$.	Before $NaHCO_3$.	After $NaHCO_3$.							
		kg.	*mm. Hg*	*gm. per liter*	*per cent*	*pH*	*pH*	*vol. per cent*	*vol. per cent*	*gm.*	*gm. per kg.*	*hrs.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
1		90				6.3	7.1	74.7	76.1	4	0.044	1	1.7	1.4	+0.3	Normal.
2		73				6.8	7.1	62.0	64.3	4	0.055	1	2.1	2.3	−0.2	"
3	PH 40433	61				6.4	7.6	70.9	71.6	4	0.066	1	2.5	0.7	+1.8	Chronic cardiac valvular disease, mitral disease, cardiac insufficiency.
4	JHH 43445	59	$\frac{270}{180}$	0.30	47	6.4	7.1	61.7	66.9	4	0.068	1	2.6	5.2	−2.6	Arteriosclerosis, hypertension, chronic nephritis.
5	PH 40439	91	$\frac{140}{100}$	0.40	46	5.8	6.3	61.0	61.0	7	0.079	2	2.9	0.0	+2.9	Subacute nephritis.
6	PH 40366	51				5.7	7.2	69.9	69.4	4	0.078	1	3.0	−0.5	+3.5	Chronic cardiac valvular disease, mitral disease, cardiac insufficiency.

7	PH 40204	75				5.2	5.3*	67.8	68.7	6	0.080	6	3.1	0.9	+2.2	Pneumonia.
8	PH 40176	95				5.5	6.8	70.7	76.0	8	0.084	4	3.3	5.3	−2.0	Chronic nephritis, hypertension.
9	PH 40266	59				5.4	7.2	61.1	66.8	5	0.085	1	3.2	5.7	−2.5	Cardiac insufficiency.
10		70				6.4	6.8	66.7	74.6	6	0.086	1½	3.3	7.9	−3.5	Normal.
11	PH 38054	70	135/100	0.32	38	5.8	7.3	60.4	61.4	6	0.086	1½	3.3	1.0	+2.3	Subacute nephritis.
12		41				6.9	7.6	65.3	70.9	4	0.098	1	3.7	5.6	−1.9	Empyema following pneumonia.
13	JHH 43556	40				6.3	6.7	76.0	76.3	4	0.100	1	3.8	0.3	+3.5	Diabetes insipidus.
14		36				6.3	7.3	58.4	66.3	4	0.111	1	4.2	7.9	−3.7	Chronic cardiac valvular disease, aortic disease.
15	PH 40278	57				6.9	7.6	69.7	78.8	7	0.123	4	4.7	9.1	−4.4	Diabetes mellitus, no ketonuria.
16	PH 39997	51	190/120	0.25	5	5.0	6.7	63.9	69.0	8	0.157	2	6.0	5.1	+0.9	Chronic nephritis, hypertension, arteriosclerosis.
17	PH 32084	58				5.5	6.9	59.9	60.0	10	0.172	1	6.5	0.1	+6.4	Cirrhosis of the liver.
18	JHH 43556	40				6.3	6.9	59.5	67.2	7	0.175	7	6.7	7.7	−1.0	Diabetes insipidus.
19	PH 41183	72				5.7	5.7*	67.4	70.3	13	0.181	6	6.9	2.9	−4.0	Syphilis, syphilitic aortitis.

* Nauseated and the experiment was discontinued.

TABLE I—*Concluded.*

Experiment No.	Hospital No.	Weight.	Blood pressure.	Blood urea.	Phenolsulfonephthalein excretion in 2 hrs.	Reaction of urine.		Combined CO_2 in plasma.		Amount of $NaHCO_3$ given.		Time between taking of first and second blood samples.	Calculated increase in CO_2, $\frac{38\ g.}{W.}$	Observed increase in CO_2.	Difference between calculated and observed CO_2 increase.	Remarks.
						Before $NaHCO_3$.	After $NaHCO_3$.	Before $NaHCO_3$.	After $NaHCO_3$.							
		kg.	*mm. Hg.*	*gm. per liter*	*per cent*	*pH*	*pH*	*vol. per cent*	*vol. per cent*	*gm.*	*gm. per kg.*	*hrs.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
20	PH 40268	60	$\frac{300}{145}$	0.50	0	5.2	7.0	59.1	70.0	15	0.250	2	9.5	10.9	−1.4	Chronic nephritis, hypertension, arteriosclerosis.
21	PH 40360	73				5.4	6.5	52.6	62.5	22	0.303	6	11.4	9.9	+1.5	Chronic cardiac valvular disease, mitral disease, cardiac insufficiency.
22		59				5.1	6.3	53.9	70.1	20	0.338	2	12.9	16.2	−3.3	Chronic nephritis.
23	PH 40389	89	$\frac{190}{130}$	0.62	16	5.4	6.0	45.3	72.4	31	0.347	5	13.3	27.1	+13.8	" " hypertension.
24	PH 39039	51				7.4	8.2	58.1	76.2	20	0.392	3	14.9	18.1	−3.2	Diabetes mellitus, no ketonuria.
25	PH 32304	33	$\frac{210}{145}$	0.68	10	5.4	7.3	45.3	66.9	14	0.428	1½	16.1	21.6	−5.5	Chronic nephritis.

26	RH 2961	69		2.54	0	5.3	7.0	52.0	70.0	30	0.435	3½	16.5	18.0	−1.5	Chronic nephritis.
27	PH 40457	95	260/125	3.90		5.4	5.4*	31.2	44.8	42	0.445	6	16.8	13.6	+3.2	" " hypertension, uremia.
28	JHH 43600	55	190/110	0.97	0	6.8	7.2	46.3	67.8	28	0.510	4	19.3	21.5	−2.2	" " "
29	PH 37492	89	195/140	0.95	15	5.4	5.9	50.6	67.2	46	0.517	7	19.6	16.6	+3.0	Chronic nephritis, cardiac insufficiency, hydrothorax, ascites.
30	PH 40323	91	210	0.92	9	5.5	6.8	47.9	68.1	49	0.538	7	20.5	20.2	+0.3	Chronic nephritis, uremia.
31	RH† 2941	42				5.4	5.9	32.5	61.4	37	0.880	4	33.5	28.9	+4.6	Diabetes mellitus, marked acidosis.
32	JHH 43658	95				5.6	6.0	34.5	74.2	90	0.950	9	36.0	39.7	−3.7	" " "
33	JHH 43722	43				5.7	5.8‡	41.8	81.5	60	1.395	5	53.0	39.7	+13.3	" " "
Average									68.7							

† Cases taken from the cases reported by Palmer and Van Slyke (1).

‡ At midnight the pH of the urine was 8.2.

DISCUSSION.

The results in Table I clearly demonstrate the reliability of employing the early effect of alkali administration on the pH of the urine as an indication of the restoration of the bicarbonate level of the blood plasma to within normal limits. A variety of pathological cases, particularly the clinical conditions known to have impaired renal function and diminished alkali reserve, were studied. For the thirty-three cases the average plasma bicarbonate CO_2 at which an appreciable depression of the acidity of the urine occurs is 68.7 volumes per cent, a value well within normal limits. The average change in the pH was 0.97. Disregarding for the moment Case 33 and Cases 19 and 27 which were discontinued on account of nausea, the maximum plasma bicarbonate CO_2 at which depression of urinary acidity occurs is 78.8 volumes per cent in Case 15, while the minimum plasma bicarbonate CO_2 value is 60.0 volumes per cent in Case 17, a variation of approximately ± 10 volumes per cent. It should be explained that in Case 33 large amounts of alkali were given in a relatively short time. Although the plasma bicarbonate CO_2 reached the high level of 81.5 volumes per cent the kidneys were excreting a urine the reaction of which was the same as that at the beginning of the experiment. Later, however, the urine became very alkaline, pH = 8.2. Similar phenomena have been observed in patients not reported in this paper. The evidence as far as it exists suggests that, in severe acidosis in diabetes mellitus, there is a disturbance of the acid-base regulatory mechanism of the kidneys. This disturbance of renal function appears to be temporary, and does not prevent the use of the pH as an index of sufficient alkali administration. In Cases 31 and 32, diabetes mellitus with severe acidosis, in which the alkali was given more slowly the plasma bicarbonate CO_2 was 61.4 and 74.2 volumes per cent, levels not to be considered dangerous. A safe manner of administration in instances of severe diabetic acidosis appears to be at the rate of not more than 10 gm. of sodium bicarbonate per hour if given by mouth. In the more severe grades of acidosis occurring in diabetes mellitus, the administration of large amounts of sodium bicarbonate by mouth may be unwise. The regulation of the diet in many instances may be sufficient to control the

situation. Where there is impending coma (plasma bicarbonate CO_2 below 30 volumes per cent) the introduction of sodium bicarbonate (25 gm. in 5 per cent solution) intravenously is desirable. Additional alkali may be given by mouth but should be discontinued at the first complaint of nausea.

In clinical practise the series of standard pH solutions may be eliminated. With the suitable indicators available, the urine obtained before the administration of alkali may be reserved as a guide to the degree of depression of the urinary acidity on subsequent observations. Such a procedure is reliable only if the physician is familiar with the use of the standard solution series.

The quantative relationships between the alkali retained in the pathological cases and the degree of acidosis is discussed by Palmer and Van Slyke (1). Since the rise in volume per cent of plasma bicarbonate CO_2 caused by absorption of g gm. of sodium bicarbonate is approximately $\frac{38g}{W}$, it should be possible to estimate the original plasma bicarbonate CO_2 in volumes per cent as follows, $68.7 - \frac{38g}{W}$. The level of the plasma bicarbonate CO_2 at which the urinary pH shows its first depression is 68.7 ± 10 volumes per cent. The $\pm$ 10 volumes per cent variation, therefore, excludes the use of the above method for estimating very accurately the initial plasma bicarbonate or the degree of acidosis from the amount of sodium bicarbonate given to produce an effect on the pH of the urine.

There is no constant relationship between the degree of injury to the renal function and the level of plasma bicarbonate CO_2 at which the first effect on the pH of the urine occurs following alkali administration. We have assembled the cases of nephritis, (1) in order of the severity of renal damage as shown by the phenolsulfonephthalein excretion and (2) in order of the magnitude of the blood urea, comparing the level of plasma bicarbonate CO_2 at which the urine showed the first effect of the alkali, as shown in Table II.

From the facts available there appears to be little or no parallelism between either the phenolsulfonephthalein excretion or the blood urea increase and the plasma bicarbonate CO_2 level at which the urinary acidity is decreased. Much more data are

necessary before a definite statement regarding the relationship of these renal functions can be made. In further study of this phase of the subject it would be necessary to separate the cases of nephritis into groups from the standpoint of the effect of disease on the several acid factors described by Palmer and Henderson (4). The acid factors in question were not determined in our cases.

TABLE II.

Experiment No.	Phenolsulfonephthalein excretion in 2 hrs.	Combined CO_2 in plasma after $NaHCO_3$.	Experiment No.	Blood urea.	Combined CO_2 in plasma after $NaHCO_3$.
	per cent	*vol. per cent*		*per cent*	*vol. per cent*
4	47	66.9	16	0.26	69.0
5	46	61.0	4	0.30	66.9
11	38	61.4	11	0.32	61.4
23	16	72.4	5	0.40	61.0
29	15	67.2	20	0.50	70.0
25	10	66.9	23	0.62	72.4
30	9	68.1	25	0.68	66.9
16	5	69.0	30	0.92	68.1
20	0	70.0	29	0.95	67.2
26	0	70.0	28	0.97	67.8
28	0	67.8	26	2.54	70.0

Our observations afford further confirmation of the validity and usefulness of the formula $b = \frac{38g}{W}$ in alkali therapy. In two instances, Cases 23 and 33, there occur rather wide variations (13.8 and 13.3 volumes per cent, respectively) between the calculated and observed increase in the plasma bicarbonate CO_2. The explanation for the discrepancy in Case 33 is not unlikely to be found in the incomplete absorption and distribution of the sodium bicarbonate among the body fluids resulting from too rapid administration. In Case 23 the large difference between the calculated and observed CO_2 is not clear. In the other cases the theoretical and actual increases in plasma bicarbonate CO_2 correspond with previous observations and are well within the limits of error for the experimental conditions.

SUMMARY.

1. In normal and pathological cases, following the administration of sodium bicarbonate, the plasma bicarbonate CO_2 approaches 68.7 ± 10 volumes per cent at the time the first depressing effect of the alkali on the urinary acidity is noted. The use of the first significant effect on the pH of the urine, therefore, in controlling the therapeutic use of sodium bicarbonate is safe and reliable.

2. Although the pH of the urine serves as a safe guide to the control of the therapeutic use of sodium bicarbonate, the inconstancy of the level of the plasma bicarbonate CO_2 at which a change in the pH of the urine occurs does not admit of estimating with sufficient accuracy the degree of diminished alkali reserve to be of practical value.

3. Confirmation of the observation of Palmer and Van Slyke that "absorbed sodium bicarbonate is distributed in approximate uniformity. . . . to the blood" and "the effect of a given dose in raising the plasma bicarbonate may be calculated by assuming that the body contains 700 cc. of fluid per kilo and that the bicarbonate absorbed is distributed therein uniformly" is afforded.

4. The variability among the several levels of plasma bicarbonate CO_2 at which a significant depression in the pH of the urine occurs in normal and pathological cases is probably explained by the varying degrees of injury to the acid-base regulatory functions of the kidney. There appears to be no close relationship between this function and the ability of the kidney to excrete phenolsulfonephthalein or the blood urea.

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DETERMINATION OF CHLORIDES IN BLOOD.

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At the present time the methods for the quantitation of chloride in the blood most generally used in clinical laboratories are those of Van Slyke and Donleavy (1), Rappleye (2), and Foster (3). Their methods give good results when properly performed. However, all of them are complicated by various pitfalls, inherent either in the method or in the collection of blood samples, which render the obtaining of accurate results, especially in the clinical laboratory, unduly difficult. For this reason a method has been devised which obviates these difficulties. It has the advantages that the same reagents are used for determination of chlorides in the whole blood and in the plasma. Furthermore, chlorides may be determined in blood samples collected for the analyses of the system of Folin and Wu, which is an indispensable part of the work of the clinical laboratory.

The method proposed is based on the precipitation of protein by copper hydroxide; on the precipitation of the oxalate and part, if not all, of the phosphate by an excess of calcium hydroxide; and on the titration of the chlorides by the procedure used by Rappleye. The copper hydroxide reagent employed to precipitate the protein has been successfully used by Harding and Mason (4) for the blood. The filtrates obtained by this means remain clear after the addition of the tungstic acid reagent of Folin and Wu (5).

Procedure.

The sample of blood is collected in dry potassium oxalate. Dry syringes should be used, as small amounts of water introduce a source of error, especially when small amounts of blood are collected.

Reagents.—1. 5 per cent copper sulfate solution, C.P.
2. 0.1 N sodium hydroxide.
3. Calcium hydroxide powder, C.P.
4. Standard silver nitrate solution:

Silver nitrate	7.2653 gm.
Nitric acid (concentrated)	150 cc.
Distilled water to	1,000 "

(1 cc. of silver solution = 2.5 mg. of NaCl)

5. 10 per cent iron alum solution.
6. Potassium thiocyanate solution: Make 1.6 gm. of potassium thiocyanate up to approximately 800 cc. and determine its strength by titrating against the silver nitrate solution. Then dilute the potassium thiocyanate solution until 12.5 cc. of it react with 5 cc. of the standard silver nitrate solution. If the precipitating reagent contains traces of chlorides then a correction must be applied to the potassium thiocyanate solution. (Most of the chemically pure articles on the market are sufficiently pure to use without making corrections.) (1 cc. of thiocyanate solution = 1.0 mg. of NaCl.)

Precipitation in the Plasma.

In a 50 cc. volumetric flask place 2 cc. of plasma accurately measured, 15 cc. of water, 4 cc. of the copper sulfate solution, and 10 cc. of 0.1 N NaOH. Heat in boiling water for about 1 minute. Keep the contents of the flask rotating while heating to insure complete precipitation of the protein. Cool the flask in running water and make up to 50 cc. mark. Shake thoroughly and filter through a chloride-free filter paper. To about 35 cc. of the filtrate add 0.5 gm. of calcium hydroxide powder and shake a few times. Allow to stand 1 minute and filter.

Precipitation in Whole Blood.

In a 50 cc. volumetric flask place 2 cc. of whole blood accurately measured, 10 cc. of water, 8 cc. of the copper sulfate solution, and 20 cc. of 0.1 N NaOH. Heat in boiling water for about 1 minute. Keep the contents of the flask rotating while heating to insure complete precipitation of the protein. Cool the flask in running water and make up to 50 cc. mark. Shake thoroughly and filter through a chloride-free filter paper. To about 35 cc. of the filtrate add 0.5 gm. of calcium hydroxide powder and shake a few times. Allow to stand 1 minute and filter.

Determination.

To 25 cc. of the filtrate from either whole blood or plasma add 5 cc. of the standard silver nitrate solution, 2 cc. of the iron alum solution, and stir until the silver chloride separates out. Titrate the excess silver nitrate with the potassium thiocyanate solution. The first reddish tinge is the end-point.

Subtract the number of cc. of potassium thiocyanate used in the titration from 12.5; the difference will be the gm. of NaCl per 1,000 cc. of whole blood or plasma.

DISCUSSION.

By the use of the method described above, sodium chloride was recovered quantitatively after its addition to the precipitating reagent. It was also quantitatively recovered after its addition to whole blood or plasma, as is shown in Tables I and II.

TABLE I.

Quantitative Recovery of NaCl Added to Plasma.

No. of sample.	NaCl in plasma.	NaCl added.	NaCl recovered.	Theoretical value.
	gm.	*gm.*	*gm.*	*gm.*
1	5.65	0.50	6.15	6.15
2	5.70	0.50	6.20	6.20
3	5.90	1.00	6.85	6.90
4	5.55	1.50	7.05	7.05
5	5.80	0.50	6.25	6.30

TABLE II.

Quantitative Recovery of NaCl Added to Whole Blood.

No. of sample.	NaCl in whole blood.	NaCl added.	NaCl recovered.	Theoretical value.
	gm.	*gm.*	*gm.*	*gm.*
1	4.65	0.50	5.15	5.15
2	4.50	1.00	5.45	5.50
3	4.55	1.50	6.05	6.05
4	4.70	0.50	5.20	5.20
5	4.30	0.50	4.75	4.80

Results obtained by the method described check with those obtained by the Austin-Van Slyke method for whole blood, and the Van Slyke-Donleavy method for plasma, as is shown by Table III.

TABLE III.

Comparison of Results Obtained with Methods of Van Slyke and the Author.

NaCl in whole blood.		NaCl in plasma.	
Van Slyke-Austin.	Author.	Van Slyke-Donleavy.	Author.
gm.	*gm.*	*gm.*	*gm.*
4.65	4.60	5.65	5.65
4.30	4.30	5.70	5.65
4.60	4.55	5.80	5.85
4.50	4.50	5.50	5.50
4.60	4.55	5.95	5.90

To check further the accuracy of the method the chloride content of whole blood and plasma was determined by a modification of the Gutmann and Schlesinger method (6) devised by the author. The method is carried out in the following manner.

In a small crucible place 1 cc. of the substance under examination and add 0.5 gm. of sodium carbonate dissolved in a small amount of water. Evaporate to dryness on a water bath or in a hot air oven. The destruction of the organic matter is carried out slowly with a microburner. It is well at short intervals to allow the crucible to cool and add a few drops of distilled water to the residue; this helps to destroy the carbonized matter without excessive heating. When the organic matter is completely destroyed the crucible is transferred to a 200 cc. beaker and a small amount of hot distilled water added. To the contents of the beaker add nitric acid until effervescence ceases. The crucible is held over the top of the beaker with a pair of tongs and washed thoroughly with distilled water. The contents of the beaker are filtered through a chloride-free filter paper, the beaker and filter paper being washed several times with hot distilled water.

To the filtrate add 5 cc. of the standard silver nitrate solution and 2 cc. of the iron alum solution; stir until the silver chloride separates out. Titrate the excess silver nitrate with the potassium thiocyanate solution.

The results obtained by the Gutmann and Schlesinger method and the method here described are shown in Table IV.

Results obtained in plasma by the method described check with those obtained by the use of the methods of Van Slyke-

Donleavy, and of Rappleye, provided the plasma samples are free from hemolysis. In the presence of even a slight degree of hemolysis results obtained by the latter two methods are of questionable accuracy, since the values for the chlorides are often too great. Van Slyke states in connection with his method for whole blood that only a protein-free filtrate is to be used for determining the chloride content, as some component of the laked cells other than the chloride combines with some of the silver nitrate, making the results too high. The same may be said of the Van Slyke-Donleavy method for chlorides in the

TABLE IV.

Comparison of Results Obtained with Methods of Gutmann and Schlesinger and the Author.

Whole blood.		Plasma.	
Gutmann and Schlesinger.	Author.	Gutmann and Schlesinger.	Author.
gm.	*gm.*	*gm.*	*gm.*
4.75	4.80	5.85	5.90
4.65	4.70	6.00	6.00
3.85	3.90	5.00	5.00
4.60	4.60	6.00	6.05

plasma when the latter is slightly colored from hemolysis. Hemolyzed plasma does not affect the accuracy of the results obtained by the method here described.

A method for the determination of chlorides in the blood has been devised by Harding and Mason (4) which is somewhat similar to the method here described. The chief objection to their method is the titration with potassium iodide, which in the author's experience is a more difficult procedure for the average clinical laboratory worker than is the titration with potassium thiocyanate.

The normal chloride threshold has been set by McLean (7) at 5.62. By the author's method normal plasmas have varied from 5.65 to 6.00.

The method described can be modified by using potassium thiocyanate, each cc. of which contains 0.5 mg. of sodium chloride; however, the end-point is not so clear as when the stronger thiocyanate is used.

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VITAMINE STUDIES.

VI. THE INFLUENCE OF DIET OF THE COW UPON THE NUTRITIVE AND ANTISCORBUTIC PROPERTIES OF COW'S MILK.*

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During the winter of 1918 experiments were in progress, in this laboratory, in which guinea pigs were fed a basal diet of 20 cc. of autoclaved milk and oats *ad libitum*. The milk used in this study was obtained from the University dairy herd which had received a grain mixture consisting of corn, barley, oats, middlings, bran, and oilmeal. The roughage consisted of silage and a poor grade of alfalfa hay. The milk was fed to the guinea pigs with the view of supplementing the oat proteins and salts, and was autoclaved to lower the antiscorbutic potency of the milk. It was found that guinea pigs which received the oats and autoclaved milk developed scurvy and died in the time usually expected on this type of diet.

However, during the spring months of 1919, it was observed that some of the animals which had been placed upon experiment late in the winter had ceased to lose in weight, scurvy symptoms were less pronounced, and the animals presented a much improved physical appearance. New groups of animals lived longer on the basal diet containing 20 cc. of autoclaved spring milk than animals receiving the same quantity of autoclaved winter milk. While scurvy developed in the new groups of animals during the spring, the symptoms were less severe and many of the animals actually

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gained in weight when a loss of weight and death would have been expected.

Inquiry revealed the fact that the University herd had been turned out to spring pasture about 10 days before we had begun to notice the improved condition of the experimental animals just described. In view of the fact that the antiscorbutic vitamine is supposed to be very susceptible to heat, we postulated at the time[1] that the improved nutritive properties of the milk were due, in all probability, to an increased amount of the fat-soluble vitamine in the milk; the last mentioned vitamine having shown greater stability toward heat than either the antineuritic or antiscorbutic vitamines. Consequently, experiments were immediately initiated using 20 and 30 cc. of whole and skimmed raw summer milk. It was found that 20 and 30 cc. of summer milk protected guinea pigs from scurvy for 90 to 100 days when other investigators had often failed to obtain protection with much larger quantities of milk. We were convinced, therefore, that the disagreements which had existed in previous studies, where milk had been fed as the antiscorbutic material, could be explained upon dietary grounds and that the vitamine content of milk is dependent upon the vitamine content of the diet of the lactating animal.

Similar suggestions had already been made by other investigators, but in the majority of cases these suggestions had not been based upon experimental evidence. Previous to our announcement,[1] it had been observed that the antineuritic vitamine in human milk is dependent upon the diet of the nursing mother.[2] McCollum and coworkers,[3] experimenting with rats, came to the same conclusion. The last mentioned experiments, while undoubtedly correctly interpreted, appear somewhat lacking in concrete proof, due to the fact that it was not possible to determine whether or not the milk flow of the experimental mother rats was sufficient for the dietary needs of the young. Obviously, experiments which involve the use of dairy cattle are much to be preferred, in this regard, for although milk production may fall (as

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it did in the study described in this paper) the amount of milk obtained is amply sufficient for feeding in any desired quantity. It is also evident that results obtained by the use of dairy cows should also be of much more practical significance. Osborne and Mendel describe experiments[4] in which they were unable to find that summer milk was superior to winter milk as far as the amount of water-soluble (B) vitamine is concerned. It has been pointed out[5] that the average winter ration is undoubtedly fairly adequate with regard to this vitamine due to its general distribution in the grains and also due to the fact that it does not seem to be injured appreciably during the drying and curing process. There is reason to believe, however, that if rations are fed which are sufficiently poor in this vitamine, the milk will also prove to be equally deficient.

During the same month (August) that we announced our observations regarding the nutritive superiority of summer milk, Barnes and Hume[6] reported that they had also noted a seasonal variation in the antiscorbutic properties of cow's milk.

In order that we might obtain more conclusive experimental evidence concerning the influence of diet upon the vitamine content of milk, we have fed vitamine-poor and vitamine-rich rations to dairy cows and the milk obtained from these animals has been subjected to investigation by means of laboratory animals. We have included in this study a biological investigation of the fat-soluble, the water-soluble (B), and antiscorbutic vitamines. In this paper we shall describe our experiments with regard to the antiscorbutic properties of milk produced under conditions of low and high vitamine feeding. In a later paper we shall submit data to show that the fat-soluble vitamine is markedly influenced by the diet of the cow, while the same is true for the water-soluble vitamine but to a lesser degree.

While our experiments were under way Hart, Steenbock, and Ellis[5] published the results of their studies of the same problem. These authors had an unusual opportunity to study this phase of nutrition inasmuch as there was available, at the University of

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[6] Barnes, R. E., and Hume, E. M., *Lancet*, 1919, ii, 323.

Wisconsin, a herd of eighteen dairy cows all of which had been "kept year after year on air-dried roughages and grains." It was found that the milk obtained from this herd was much inferior, in antiscorbutic properties, to summer milk *derived from other cows* in the University herd.

The experiments described in this paper differ from the Wisconsin study inasmuch as *the milk was derived from the same cows throughout the experiment, thereby eliminating any possible question of differences in milk from individual cows.* We have attempted, also, to obtain information regarding the length of time required for the vitamine-poor and vitamine-rich rations to produce an effect upon the nutritive properties of the milk. This has been done by adding new groups of guinea pigs from time to time as the experiment progressed.

EXPERIMENTAL.

Period 1; the Vitamine-Poor Period.

The Cows.—Two cows (a Jersey and a Holstein) were fed a vitamine-poor ration for a period of 4½ months (January 17th, 1920, to June 1st). From June 1st to October 1st (4 months) they received the same grain ration and were given constant access to pasture grass. The Jersey and Holstein breeds were chosen on account of the fact that the former produces a milk with a high percentage of butter fat, while the Holstein milk is characterized by a relatively low content of butter fat. By mixing these milks it was thought that the resulting mixture would be fairly representative of average herd milk in chemical composition. Both of these animals had calved during the latter part of December, and were placed upon the vitamine-poor ration on the 17th day of the following month. The cows were milked morning and evening and samples of the mixed milk for the 24 hours were taken for the feeding experiments.

The Ration.—The constituents of the vitamine-poor ration were selected to satisfy the nutrient requirements of the animals and at the same time maintain a low supply of the fat-soluble and antiscorbutic vitamines.

The supply of the water-soluble (B) vitamine was kept as low as was practicable without going outside of the list of ordinary

cattle feeds, by the use of gluten feed (as a high protein feed), wheat middlings, and straw. The animals received what they would consume of a roughage consisting of equal parts of chopped timothy hay and oat straw. The grain rations consisted of equal parts of wheat middlings, gluten feed, ground oats, and ground barley. The ration was adjusted, in quantity, to satisfy the requirements of the animals, according to Armsby's feeding standard. While this ration is undoubtedly poor in the fat-soluble and antiscorbutic vitamines, on the whole, it is a better ration than is often fed on many farms during the winter months, and a fair flow of milk was obtained during the period that it was fed.

Records were kept of feed consumed and of milk produced, while fat tests were made at regular intervals throughout the experiment. On June 1st the cows were changed abruptly to pasture, while the feeding of the same grain ration was continued but in smaller amounts.

The Guinea Pigs.—Healthy, normal guinea pigs were used in all the feeding experiments. Most of the guinea pigs weighed between 200 and 300 gm.; only in a few cases did we use animals weighing as much as 400 gm. All the experimental groups contained at least three (and often four) guinea pigs which were chosen with regard to body weight. Each group contained one heavy animal, one light animal, and one or two which fell between these two extremes. This method placed all the groups of animals on a comparative basis, in case the size of the animal should become an influencing factor in the development of scurvy. Each animal was confined in a cage by itself and the cages were cleaned twice each week and washed with hot water. The cages were sprayed with a cresol preparation at each cleaning period.

Feeding.—The guinea pigs were allowed all the oats that they would eat. The milk, which was fed in varying amounts, was fed twice daily in clean porcelain dishes, which were introduced at each feeding. The animals were under observation at all times and weighings were made every 5 days until scurvy symptoms appeared, and at 3 day intervals thereafter.

We have described the symptomatology and postmortem appearances in guinea pig scurvy in a previous publication.[7]

[7] Dutcher, R. A., Pierson, E. M., and Biester, A., *J. Biol. Chem.*, 1920, xlii, 301.

On February 1st (2 weeks after the cows had been placed on the vitamine-poor diet), five groups of guinea pigs (totaling twenty animals) were placed upon experiment. The quantities of milk fed to the respective groups were as follows: 30, 40, 50, 70, and 80 cc.

On March 1st (1 month later), a second series of animals was placed upon the oats and winter milk diet in order to ascertain, if possible, whether or not the milk was becoming poorer in nutritive properties as the experiment progressed. This series consisted of five groups of guinea pigs (totaling fifteen animals). These groups received the following amounts of milk respectively: 20, 30, 40, 50, and 60 cc.

The third series of guinea pigs was started on April 1st (2 months later). This series was also made up of five groups of three animals each (totaling fifteen animals). These groups were not fed the larger quantities of milk on account of the short time remaining before the cows were to be turned out to pasture. The amounts of milk fed to the respective groups in this series were as follows:

Group 1, 20 cc. Jersey milk.
" 2, 30 " " "
" 3, 20 " Holstein "
" 4, 30 " " "
" 5, 30 " mixed "

The Jersey and Holstein milks were fed separately at this point to determine whether or not breed differences existed with regard to the nutritive properties of winter milk.

Period 2. The Vitamine-Rich Period.

On June 1st the cows were changed abruptly to pasture and the grain ration was continued. Three groups of guinea pigs (four pigs to the group) were placed upon experiment on June 1st, the day that the cows were given access to grass. These groups were fed 20, 30, and 40 cc. respectively of the mixed summer milk. 2 months later (August 1st) three additional groups of guinea pigs were added. These also received 20, 30, and 40 cc. of milk per animal in the respective groups. Observations and weighing were made as in Period 1.

DISCUSSION.

Examination of the data concerning the groups of guinea pigs receiving 20 cc. of milk (Chart 1) shows quite graphically the superiority of the summer milk over that produced in the winter on the vitamine-poor ration. In the remainder of this paper we shall use the terms "winter milk" and "summer milk" to differentiate the milk produced on the vitamine-poor and vitamine-rich rations. All the animals (Nos. 120, 121, and 122) which were placed on the winter milk on March 1st died within 10 weeks with marked evidence of scurvy. (S = first scurvy symptoms observed.) Two of the animals (Nos. 135 and 136) which were started on April 1st died with scurvy, while No. 137 lived on into Period 2 and responded to the summer milk almost immediately. Animals 135, 136, and 137 were fed Jersey milk, while Animals 141, 142, and 143 were fed Holstein milk. All the other animals in Chart 1 were fed mixed milk. No breed differences are apparent in these two groups. It should be noted that Animals 137 and 142 (which showed improvement after beginning the summer milk) had shown pronounced scurvy symptoms in Period 1, and when autopsied on the 1st of August no pronounced scurvy symptoms or lesions could be observed.

20 cc. of summer milk protected Animals 200, 201, and 203 for periods of 16, 4, and 8 weeks respectively, while of the four animals which were placed on summer milk in August (Nos. 260, 261, 262, and 263) three remained healthy and normal throughout the 8 weeks that they were on experiment.

Much to our surprise the first groups of animals (Nos. 103, 104, and 105), which received 30 cc. of winter milk (Chart 2), did not die or fall off in body weight although mild scurvy symptoms developed. However, Animals 123, 124, and 125 developed scurvy and died. No outstanding difference can be noted in the group receiving Jersey milk (Nos. 147, 148, and 149) and those receiving the same quantity of Holstein milk (Nos. 144, 145, and 146). Nos. 139 and 145 responded very soon (2 weeks) when changed to summer milk. Animals 204, 205, 206, and 207 which were started on summer milk on June 1st remained in excellent condition and did not develop scurvy throughout the 16 weeks feeding period, and the same holds true for Animals 264, 265, and 267

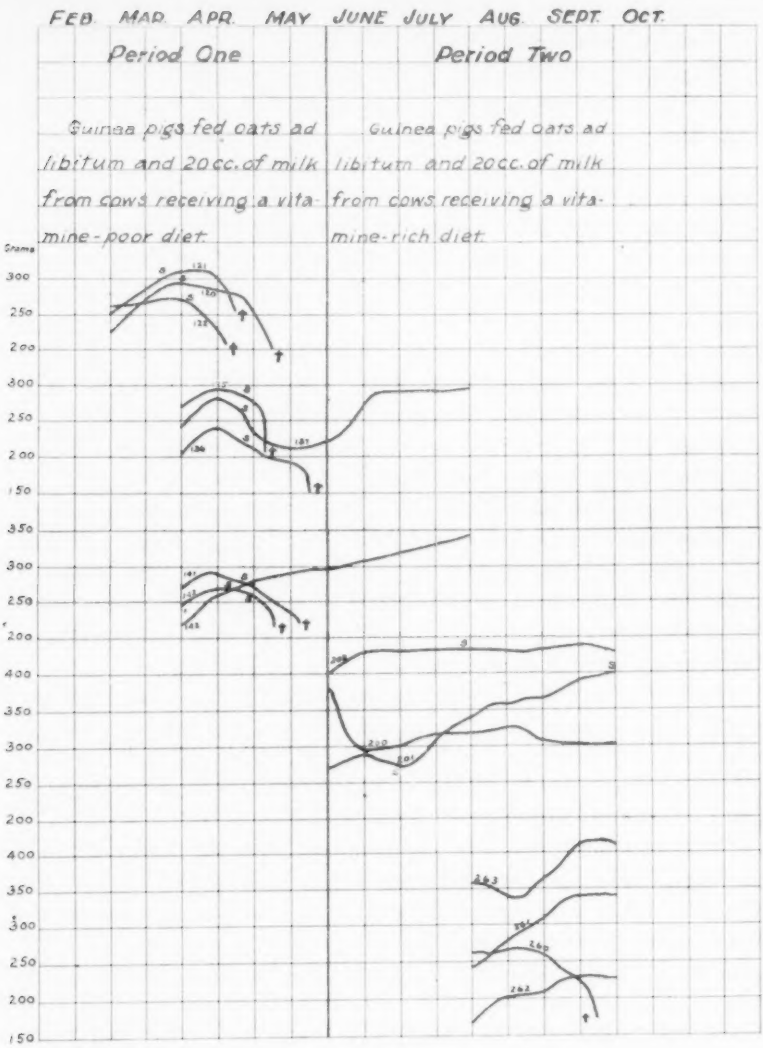
FEB. MAR. APR. MAY JUNE JULY AUG. SEPT. OCT.
Period One
Period Two
Guinea pigs fed oats ad libitum and 20 cc. of milk from cows receiving a vitamine-poor diet.
Guinea pigs fed oats ad libitum and 20 cc. of milk from cows receiving a vitamine-rich diet.
Grams

CHART 1. Showing the superiority of 20 cc. of summer milk over the same quantity of winter milk.

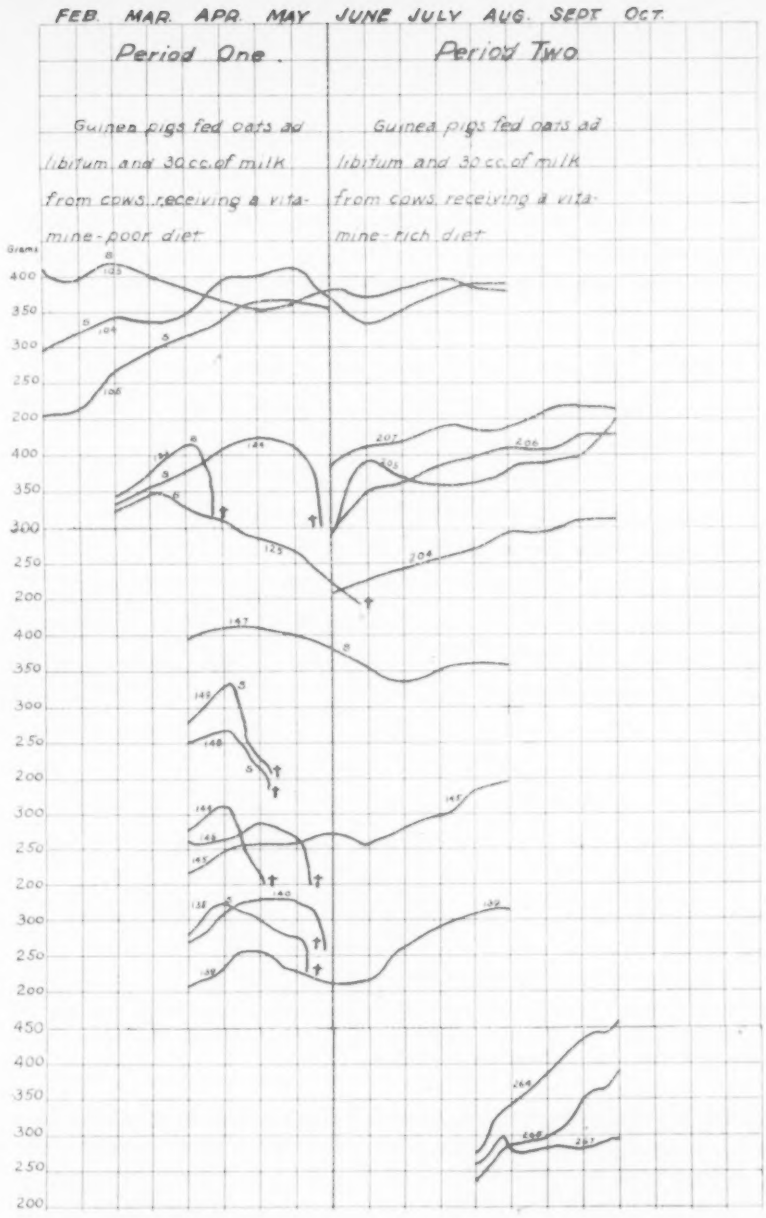
FEB. MAR. APR. MAY JUNE JULY AUG. SEPT. OCT.
Period One.
Guinea pigs fed oats ad libitum and 30 cc. of milk from cows receiving a vitamine-poor diet
Period Two.
Guinea pigs fed oats ad libitum and 30 cc. of milk from cows receiving a vitamine-rich diet
Grams

CHART 2. The group which was placed upon experiment in February grew well on 30 cc. of milk. 1 month later the milk had become so poor that all the animals died. 2 months later the milk was of such poor quality that six of the nine experimental animals died. Those which survived improved in physical appearance and showed fair growth when 30 cc. of summer milk were fed. All the animals receiving 30 cc. of summer milk grew well and no scurvy symptoms developed.

which were started on summer milk on August 1st. The superiority of the summer milk is quite evident from the curves of the last mentioned groups.

The animals which received 40 cc. of milk reacted in practically the same manner as the 30 cc. groups (see Chart 3). Those which received winter milk beginning February 1st grew well and did not develop scurvy. This might be explained upon the assump-

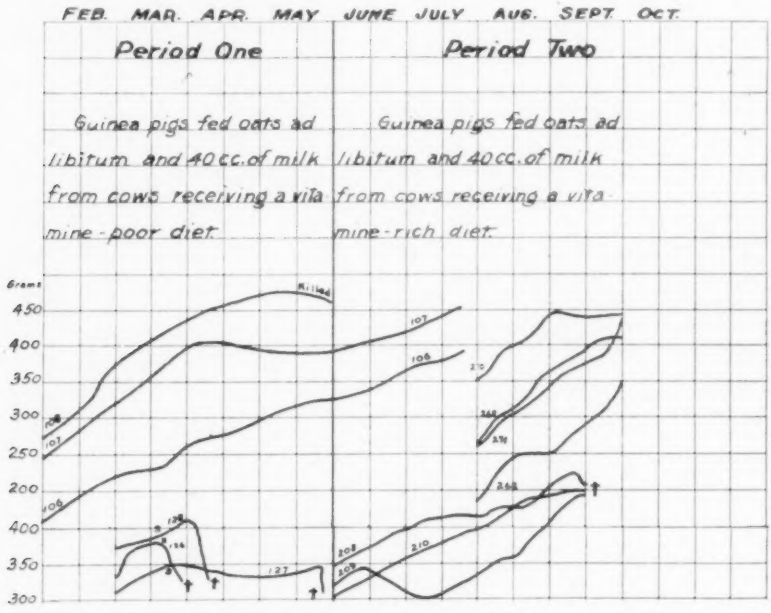

CHART 3. All animals placed on experiment in February lived and grew well. 1 month later the winter milk was of such poor quality that all the animals died. All the animals receiving 40 cc. of summer milk grew well and did not develop scurvy symptoms.

tion that the cow and the guinea pig, together, stored sufficient vitamines to carry the guinea pigs over the winter period. The fact that the March and April groups did not do so well probably lies in the fact that the milk was becoming progressively poorer in vitamines, and after the supply stored by the guinea pig was exhausted the deficiency disease developed. The groups receiving 40 cc. of summer milk grew well and did not develop scurvy.

No. 208 died suddenly during September having developed no scurvy symptoms. The cause of death is unknown.

The February groups of guinea pigs receiving 50 cc. of milk grew well and did not develop scurvy (see Chart 4). Animal 110

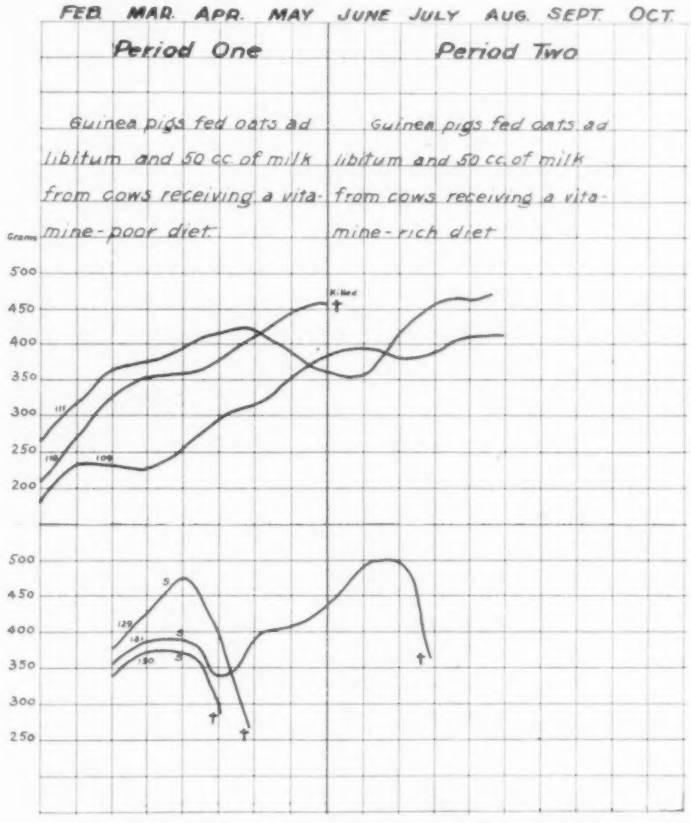

CHART 4. 50 cc. of winter milk did not protect the animals against the development of scurvy and all the animals died with the exception of the group which was placed on diet before sufficient time had elapsed for the milk to become poor.

was killed on June 1st and was found to be in excellent physical condition. The group which was started March 1st (1 month later) developed scurvy and died. Animal 131 did not respond

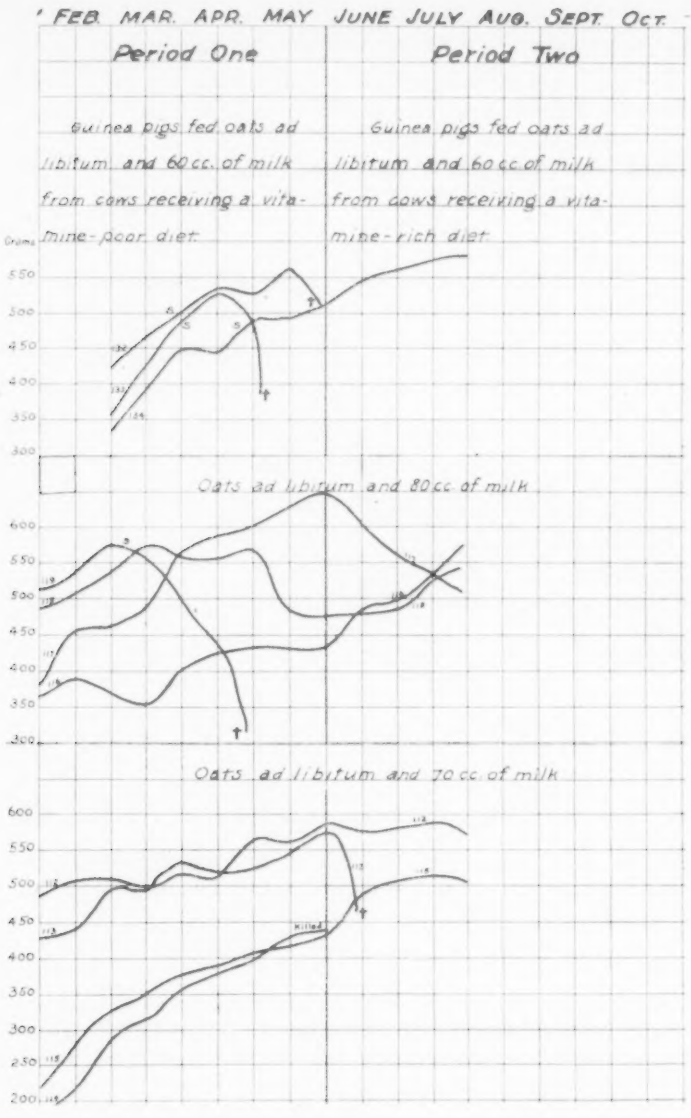
FEB. MAR. APR. MAY JUNE JULY AUG. SEPT. OCT.
Period One
Period Two
Guinea pigs fed oats ad libitum and 60 cc. of milk from cows receiving a vitamine-poor diet.
Guinea pigs fed oats ad libitum and 60 cc of milk from cows receiving a vitamine-rich diet.
Oats ad libitum and 80 cc of milk
Oats ad libitum and 70 cc of milk
Killed

CHART 5. All guinea pigs receiving 60 cc. of winter milk developed scurvy and two of the animals died. After 2 months on a diet of 60 cc. ofsummer milk no scurvy symptoms could be detected in the animal which survived the winter period, although scurvy symptoms had been very pronounced in the winter months.

to the summer milk and died with very pronounced scurvy lesions. Chart 5 shows that 60 cc. of the "late" winter milk did not protect the animals against the development of scurvy. The scurvy symptoms (swollen joints) disappeared, in the case of No. 134, after 60 cc. of winter milk were replaced by 60 cc. of summer milk.

None of the groups receiving 70 cc. of winter milk (Chart 5), beginning the 1st of February, developed scurvy. The same may be said for the 80 cc. group (Chart 5) with the exception of Animal 119 which did not take the full amount of milk at the beginning of the feeding period.

When the charts are compared as a whole, it is evident that 20 cc. of summer milk were superior in nutritive value and in antiscorbutic potency to 60 cc. of winter milk. A study of the winter milk groups seems to show that the milk does not become poor rapidly; in fact, it is not until the 5th to the 8th week that the effect of the diet becomes manifest in the growth curves and physical condition of the experimental animals. On the other hand, it seems equally certain that the effect of the vitamine-rich ration is noticeable within 2 weeks after the cows were turned out to pasture. This is rather to be expected for it is but natural that the mother will draw upon the vitamines stored in her tissues in order to protect the offspring, even to the sacrifice of her own physical well being. Nor is it surprising to find that the milk becomes rich in vitamines almost immediately upon the ingestion of a vitamine-rich diet.

SUMMARY AND CONCLUSIONS.

Two cows, a Jersey and a Holstein, were placed upon a vitamine-poor ration in the middle of January. Mixed milk was fed in varying quantities to several groups of guinea pigs, new groups of guinea pigs being added from time to time as the experiment progressed. Beginning June 1st the cows were given a vitamine-rich ration and the former experiment was repeated. From the data obtained it was concluded that the vitamine content of cow's milk is dependent upon the vitamine content of the ration ingested by the cow. 20 cc. of summer milk were superior in nutritive value and in antiscorbutic potency to 60 cc. of winter milk. It was found that there is a tendency for the milk to become poor

slowly, when the diet of the cow is low in vitamines, while the milk becomes of higher nutritive value almost immediately upon the ingestion of a vitamine-rich ration. In other words, there seems to be a tendency for milk to become poor slowly and rich rapidly as far as the vitamines are concerned.

In conclusion, the writers wish to acknowledge the assistance of Mr. D. Bessessen in performing a number of the autopsies recorded in this paper.

ABSORPTION AND ELIMINATION OF MANGANESE INGESTED AS OXIDES AND SILICATES.

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In 1919 Edsall, Wilbur, and Drinker (1) reviewed the older literature and described cases of manganese poisoning in the United States, occurring among mill employees who were working in a dust containing manganese as oxides and silicates. They point out that the chief portals of entry for the dust are the gastro-intestinal tract and the lungs. Up to the present time, owing to lack of reliable methods of analysis, these clinical findings have never been supplemented by adequate analytical data on the manganese content of blood, tissues, or excreta. With the development of the method for detection and quantitation of minute amounts of manganese in biological material, published recently from this laboratory (2), the following experiments were carried out to study the absorption, distribution, and excretion of ingested manganese in the form of oxides and silicates.

Solubility in Gastric Juice.

The extent to which swallowing a manganese-containing ore involves a possible risk depends primarily upon its solubility in the gastric juice. This point was tested with franklinite, rhodenite, and manganese dioxide, representative manganese ores.[1]

[1] Franklinite is a mixed oxide of manganese, iron, and zinc. Analysis of the specimen used by us shows manganese 9.49, zinc 14.53, iron 27.87, calcium oxide 10.74, magnesium oxide, 1.07, silicon dioxide 5.79, and carbon dioxide 8.30 per cent. Rhodenite when pure is manganese silicate. In the sample employed calcite was the chief impurity and the ore contained 24 per cent of manganese as silicate. Chemically pure manganese dioxide containing 48 per cent of manganese was used.

1 gm. of the various ores, ground to 200 mesh, was incubated for different lengths of time in 50 cc. of fasting stomach contents obtained in the usual manner from hospital patients. In one case, in order to approach more nearly to normal conditions, C. K. D. took 8 gm. of franklinite with a test meal which was removed and the stomach washed after 30 minutes. Filtration and analysis were done without incubation. The complete removal of the finely divided dust from a water suspension by filtration proved difficult. The filter finally used was repeatedly proved to be efficient and consisted of an alundum thimble which had been previously dipped in collodion and dried. This was encased in a brass chamber and filtration carried out under about 60 pounds pressure.

A few typical figures for solubilities found are as follows: 14.13 mg. of manganese dissolved after 96 hours at 37°C. in juice with total acidity of 74; 5.56 mg. of manganese dissolved after 22 hours at 37°C. in juice with total acidity of 40; 1.10 mg. of manganese dissolved, in case of test meal (C. K. D.), in contents with total acidity of 21. Unquestionably the ores tested are soluble in gastric juice to an extent depending upon the acidity and duration of contact, but sufficient under normal conditions so that workmen who continually swallow small amounts of such ore dusts must continually furnish dissolved manganese for absorption into the blood stream.

Since the various ores showed practically the same solubility, franklinite was used in most of our feeding experiments.

Effect of Ingested Manganese Ores on the Manganese Content of the Blood.

We were fortunate in being able to obtain blood specimens from employees of the New Jersey Zinc Company at Franklin, New Jersey.[2] Before the installation of the present dust removal systems and methods of periodical physical examination of employees, several cases of manganese poisoning had occurred at this plant. The blood samples were taken by one of us, choosing as subjects some of the men who had been in the employ of the

[2] This company very kindly further cooperated with us by supplying and preparing the franklinite and rhodenite used in these experiments.

company for a long time and whose work kept them a part of the time in some of the dusty places, such as the bag house connected with the dust removal system. The blood was removed from one of the large veins of the arm during the working hours of the men, employing an all glass syringe and using the greatest care to prevent contamination. The figures obtained by analysis are presented in Table I.

TABLE I.

*Manganese Content of Bloods of Franklin Employees.**

Name.	Mn per 100 gm. blood.	Name.	Mn per 100 gm. blood.
	mg.		*mg.*
S. D.	0.008	T. F.	0.010
J. W.	0.008	R. L. McC.	0.005
P. H.	0.019	J. B.	0.021
J. P.	0.013	W. A. M.	0.016
C. S.	0.005	M. E. P.	0.023
O. G.	0.009	W. D.	0.016
G. B.	0.020	H. S.	0.010
C. C.	0.010	A. M.	0.006
L. B. D.	0.013	R. D.	0.006
H. V. A.	0.023	W. C.	0.010
R. M.	0.006	G. M.	0.022
C. D.	0.019	J. J.	0.020
C. B.	0.013	L. B.	0.016
J. K.	0.010		
Average			0.013

* 30 gm. samples of blood were analyzed in every case.

We had hoped to find variations from the normal content which at least in the case of certain individuals would be sufficiently marked to show a connection between the manganese concentration in the blood and susceptibility to poisoning. A study of the results, however, shows that in no case is the concentration unusually high, and that the mean result as well as the variations from the mean are very close to the normal figure (0.012 mg. per 100 gm. of blood) reported in an earlier paper (2). In view of findings to be reported later in this paper this is not surprising. In the case of the workmen whose bloods have been studied the intake of manganese, though continuous, is very small.

and the normal rate of excretion, as will be shown later, is more than adequate to keep the concentration in the blood at a normal level.

This leads directly to the question of whether larger amounts of ore dust taken at one dose may not temporarily increase the manganese of the blood to a higher level. To prove this point, volunteers in the laboratory submitted to the following experiment which in several instances was repeated with identical results. The subjects in every case drank a water suspension containing 8 gm. of franklinite. Analyses were made of blood samples taken before and 1, 3, 6, and 24 hours after taking the ore. In the experiment of September, 1920, in order to show whether conditions tending to affect the acidity of the gastric juice would alter the amount of manganese in the blood, 50 gm. of cooked meat were eaten immediately after swallowing the ore dust. As these results are in no way different from those of previous experiments when the dust was taken without food, the data are all presented together in Table II.

In almost every case the manganese concentration increased slightly during the first hour or two and then fell rapidly back to normal. It is apparent that one or more of the following three factors tends to maintain this low level: (1) the rate of solution of the ore by the gastric juice is very slow, or (2) the rate of absorption into the blood stream is equally slow, or (3) the rate of removal from the blood is too rapid to allow any considerable rise of the manganese content of the blood. The literature upon the behavior of foreign crystalloids in the blood stream and experiments of our own in which soluble manganese has been injected into the blood stream cause us to favor the last possibility as the controlling factor, since a few minutes after a considerable intravenous injection of manganous chloride the manganese content of the blood is practically normal.

Whatever the controlling factor, since identical results have repeatedly been obtained in the same subject and varying results with different individuals, it is evident that in some subjects the manganese content of the blood can temporarily be doubled by ingestion of manganese compounds while in others the concentration remains constant. It seems probable that individuals falling into the first group would be more susceptible to man-

ganese poisoning than those in whom the concentration does not rise.

TABLE II.

*Manganese Concentration of Blood after Eating Franklinite.**

Name.	Date.	Mn per 100 gm.				
		Control.	1 hr. after.	3 hrs. after.	6 hrs. after.	24 hrs. after.
	1919	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
C. K. D.	Dec. 19	0.012	0.024	0.017	0.019	0.014
	1920					
"	Jan.	0.011	0.023	0.010		
"	Sept.	0.010	0.022	0.012	0.013	
C. K. R.	Jan. 20	0.008	0.017	0.010	0.010	
"	Sept.	0.010	0.028	0.021	0.013	
R. M. T.	Jan. 15	0.010	0.014	0.011	0.011	0.010
"	Sept.	0.012	0.011	0.010	0.010	
L. A. S.	Jan. 15	0.010	0.017	0.012	0.008	0.010
"	Sept.	0.008	0.013	0.010	0.010	
A. S. M.	Jan. 20	0.014	0.012	0.017	0.015	
"	Sept. 27	0.013	0.013	0.016	0.010	
W. O. F.	Jan. 15	0.011	0.010	0.011	0.010	0.011
L. F.	" 20	0.009	0.014	0.007		
W. H.	" 20	0.008	0.009	0.012	0.008	
K. R. D.	Sept. 27	0.013	0.020	0.017	0.013	
H. P. C	" 27	0.011	0.014	0.012	0.011	

* 8 gm. franklinite containing 0.77 gm. manganese eaten in every case. 30 gm. samples of blood taken in every case.

Elimination of Manganese.

The rapid return of the blood to a normal manganese content raises the question of the manner of elimination. Wichert (3), Kobert (4), Cahn (5), and Harnack (6) have stated that manganese is excreted in the bile and intestines but give very little analytical data. A more elaborate feeding experiment than those described above was carried out, in which C. K. D. ate 25 gm. of franklinite in the course of 3 days. During that time and for several days after, the total 24 hour amounts of urine and feces were collected and carefully sampled. 500 cc. of urine and 2 gm. of feces were used for analysis and the total manganese excreted in 24 hours was calculated.

Like many of the heavy metals, manganese is excreted chiefly by way of the intestines. In no case was more than a trace found in the urine, while approximately complete recovery was made in the feces (Table III), a finding which has been repeatedly corroborated by later work on both men and animals. The figures for blood in this case are remarkable only because they again show the identical level of manganese concentration reached by the same subject after eating dust in the experiment in Table II.

TABLE III.

Elimination of Ingested Manganese.

Date.	Mn per 100 gm. blood.	Total Mn in urine for 24 hrs.	Total Mn in feces for 24 hrs.	Remarks.
1919	*mg.*	*mg.*	*mg.*	
Nov. 4–5	0.012	0.000	2.59	Control.
" 5–6		0.014	1.72	"
" 6–7		0.008	5.99	"
" 7–8		0.021	12.58	5 gm. franklinite Nov. 7, 10 a.m.
" 8	0.024			5 " " " 7, 4.30 p.m.
" 8–9		0.012	748.1	Blood drawn Nov. 8, 10 a.m.
" 9–10				5 gm. franklinite Nov. 8, 10 a.m.
" 10	0.010	0.069	1,830.5	5 " " " 8, 4.30 p.m.
" 10–11				Blood drawn Nov. 9, 10 a.m.
" 11–12		0.000	772.6	Control.
" 12–13		0.000	169.9	"
		0.000	11.55	"

Following out the suggestion that manganese is eliminated from the blood stream in the bile, the occurrence of the metal in this fluid was studied after feeding franklinite to three persons with biliary fistulas. All the subjects were hospital patients with constant drainage of bile following bile duct operations. Control analyses before giving the ore show that the normal manganese content of bile is very low while after eating the ore it was very markedly increased, especially in Cases 1 and 3 (Table IV). No quantitative comparison can be made between the total amounts of eaten and excreted manganese, for only a fraction of the ore was dissolved and absorbed in the stomach and intestines and not all the bile secreted was obtained through the fistula. Never-

theless, the results do show that the bile is at least one important path of elimination of ingested and absorbed manganese.

TABLE IV.

Elimination of Manganese in Bile.

Date.	Amount ingested.	Mn per 100 gm.
	1. A. M.	
1919		*mg.*
Dec. 13	Control.	0.010
" 14	"	0.013
" 15	"	0.013
" 16	"	0.010
" 16	Ate 5 gm. franklinite	
" 17	in 3 doses.	0.111
" 18		0.100
" 19		0.054
	2. E. L.	
Feb. 16	Control.	0.003
" 16	Ate 3.3 gm. franklinite	
" 17	in one dose.	0.011
" 18		0.006
" 19		0.005
" 20		0.005
" 21		0.003
	3. M. M.	
Feb. 17	Control.	0.003
" 17	5 gm. franklinite in one	
" 18	dose.	0.108
" 19		0.120
" 20		0.046
" 21		0.027
" 22		0.033
" 23		0.018
" 24		0.018

Effect on Blood and Tissues of Prolonged Feeding of Large Amounts of Manganese Ores to Dogs.

Up to this point, the work on human subjects, while it proved the absorption and prompt elimination of manganese ore dust when swallowed, obviously could not be carried on for long

enough periods to be in any way comparable to the long continued exposure in the mills; neither has it been possible to obtain autopsy material from cases of poisoning in order to study the distribution of absorbed manganese in the body. Accordingly, animal experimentation was undertaken to obtain such data.

Six healthy dogs were chosen as subjects and for a long period they were well cared for and fed a diet of bread and meat with which had been mixed their daily ration of a manganese ore. Blood specimens were taken from the jugular vein at intervals of 1 month, and both the manganese and non-protein nitrogen content was determined. The nitrogen determinations were made by Dr. Folin by the method of Folin and Wu (7). As there was no change in nitrogen level, these figures were omitted.

Dog 6 was killed after 11 months because of poor condition due to an attack of distemper rather than to any effects that could be ascribed to manganese feeding. Dog 7 developed an extensive cellulitis and died after removal of a pus sac from his neck. The other four dogs were apparently in as good condition at the end of the experiment as at the beginning, an observation further substantiated by the fact that Dog 3 gave birth to four normal healthy puppies during the experiment.

The figures in Table V show no significant change in manganese concentration in the blood during the entire course of the experiment. The fluctuations may be explained by the fact that the specimens were taken at varying intervals after feeding, and the different values simply further illustrate the fact that the manganese content of the blood is temporarily increased after eating manganese ores. The metal must, then, either be completely eliminated or perhaps stored in some insoluble non-toxic form in certain tissues of the body.

When it became apparent that it was impossible by feeding to produce any changes demonstrable in living animals, or to produce the slightest symptom of manganese poisoning, the dogs were killed and a complete analysis of the tissues was made. A comparison of the figures in Table VI with the normal values obtained by Lund, Shaw, and Drinker (8) for various dog tissues shows that the manganese content of all tissues in the dogs fed with ores is somewhat higher, the increase being more marked in the liver and bile as the results given in Table IV would lead one

TABLE V.

Manganese in Blood of Dogs in Prolonged Feeding of Manganese-Containing Ores.

Legends.	Mar., 1919.	May, 1919.	July, 1919.	Sept., 1919.	Nov., 1919.	Jan., 1920.	Feb., 1920.	Mar., 1920.	May, 1920.	July, 1920.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Dog 1. 6.7 gm. franklinite per day. Mar. 26, 1919–July 5, 1920. 477 days. Total 3,200 gm. = 304 gm. Mn.	0.028	0.035	0.033	0.026	0.011	0.030	0.018	0.010		0.016
Dog 2. 6.7 gm. franklinite per day. Mar. 26, 1919–July 5, 1920. 477 days. Total 3.200 gm. = 304 gm. Mn.	0.017	0.022	0.022	0.020	0.036	0.042	0.019	0.023	0.016	0.024
Dog 3. 3.5 gm. Baker's 82.5 per cent MnO_2 per day. 477 days. Total 1,690 gm. = 768 gm. Mn.	0.028	0.020		0.018	0.018	0.020	0.018	0.036	0.016	0.010
Dog 5. 3.5 gm. Baker's 82.5 per cent MnO_2 per day. May 15, 1919–Mar. 30, 1920. 455 days. Total 1,450 gm. = 696 gm. Mn.	0.020		0.013	0.016	0.015	0.022	0.012	0.010	0.020	0.036
Dog 6. 3.5 gm. rhodenite per day. May 15, 1919–Mar. 30, 1920. 320 days. Total 1,120 gm. = 269 gm. Mn.		0.040	0.024	0.022	0.010	0.039	0.010	Tr.		
Dog 7. 3.5 gm. rhodenite per day. May 15, 1919–Feb. 8, 1920. 270 days. Total 950 gm. = 228 gm. Mn.			0.024	0.020	0.028	0.022	0.010			

to expect; that is, the manganese in these tissues was in the process of being eliminated into the intestine. There is no special local storing up of the metal in any organ. The chemical analyses were supplemented by histological examinations which revealed nothing abnormal in any tissue.

TABLE VI.

Manganese Content of Tissues after Prolonged Manganese Ingestion.

Form and total amount of Mn ingested	Dog 1. Franklinite. 304 gm. Mn.	Dog 2. Franklinite. 304 gm. Mn.	Dog 3. Baker's c.p. MnO_2. 708 gm. Mn.	Dog 5. Baker's c.p. MnO_2. 696 gm. Mn.	Dog 6. Rhodenite. 269 gm. Mn.	Dog 7. Rhodenite. 225 gm. Mn.	Average normal manganese content.*
Duration of experiment, *days*	477	477	477	455	320	270	
Tissue.	Mn per 100 gm.						
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Liver	0.700	0.660	0.276	0.545	0.54	0.90	0.238
Kidney	0.216	0.120	0.170	0.188	0.133	0.11	0.087
Spleen	0.025	0.112	0.032	0.043	0.046	0.031	0.022
Pancreas	0.220	0.284	0.137	0.100	0.089	0.170	
Stomach	0.115	0.211	0.081	0.710†	2.14†	0.059	0.043
Duodenum	0.377	0.088	0.051	0.203	0.175	0.072	
Small intestine	0.300	0.420	0.114	0.211	0.168	0.062	0.028
Colon	0.314	1.56	0.310	0.396	0.307	0.060	0.080
Lung	0.024	0.050	0.030	0.056	0.040	0.047	0.023
Heart	0.025	0.067	0.071	0.040	0.300	0.028	
Brain hemispheres	0.030	0.023	0.153‡	0.042	0.039	0.031	0.062§
Basal ganglia	0.042	0.046		0.138	0.057	0.049	
Cord	0.040	0.024	0.049	0.082	0.031	0.088	0.044
Omentum	0.006	0.130	0.009	0.017			
Muscle	0.020	0.042	0.025	0.023	0.016	0.034	0.020
Bone marrow	0.043	0.055	0.225	0.045			0.101
Bile	1.60	3.68		1.08	0.666	1.70	

* Lund, Shaw, and Drinker (8).
† Dog had eaten recently.
‡ Brain analyzed as a whole.
§ Whole brain.

SUMMARY AND CONCLUSIONS.

Ores containing manganese as oxides and silicates are soluble in gastric juice. Manganese is absorbed in the blood stream causing in most cases a slight temporary rise in manganese concentration followed by a quick return to normal. In none of the cases studied was the manganese content of the blood increased by the ingestion of manganese ores to a value more than double the normal level, and in some of the subjects no increase was noted. We suggest that individuals of the first group would probably be more susceptible to manganese poisoning than those of the latter.

Even prolonged feeding of large amounts of manganese ore to dogs failed to produce significant changes in manganese content of blood and tissues or to cause any pathological symptoms. Manganese ores are thus very non-toxic and in order to produce symptoms of poisoning must be ingested by individuals who are peculiarly susceptible. Clinical experience (1) has demonstrated that such persons are extremely rare.

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GROWTH ON DIETS POOR IN TRUE FATS.*

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The reason why there is no available information respecting the actual requirement of the healthy mammal for fat is attributable to the experimental difficulties heretofore inherent in its solution.[1]

In a previous paper[2] we employed the word "fat" to designate the simple glycerides of fatty acids. Attention was specifically directed to the possible independent significance of "that heterogenous group of substances designated 'lipoids'." At the same time we reported that rats even doubled or tripled their body weight upon fat-free rations consisting of a purified protein (casein or edestin), corn-starch, sucrose, and either natural protein-free milk extracted with anhydrous ether or "artificial protein-free milk" composed of a mixture of inorganic salts and lactose. Despite this apparent initial success all the rats in the reported trials and many others conducted at that time failed to reach a large size and sooner or later began to decline in weight. Some of them died at this stage; others were restored by the use of milk food containing fat. In referring to this outcome we[3] remarked that "this failure to attain complete growth involves

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

[1] A discussion of earlier attempts in this direction will be found in our monograph, Osborne, T. B., and Mendel, L. B., Feeding experiments with isolated food-substances, *Carnegie Inst. Washington, Pub. No. 156*, pts. i and ii, 1911.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1912, xii, 81.

[3] Osborne and Mendel,[2] p. 84, foot-note 10.

some factor in nutrition other than the fat and is at present under investigation."

At that time the significance of the vitamines had not been clearly formulated. It was in part the outcome of these experiments which led us to the discovery of the fat-soluble vitamine.[4] The nutritive failure of the animals in the series of 1912 was undoubtedly attributable in part at least to a deficiency in this vitamine. The fact that the foods, though extracted with ether, had not been extracted with hot alcohol, and hence could not be regarded as *lipoid*-free, may explain the not inconsiderable growth obtained, for the so called "lipoids" include substances soluble in hot alcohol which may not dissolve in ether.[5] Evidence since accumulated indicates that the so called fat-soluble vitamine is not always removed by ether unless the product has previously been treated with alcohol.[6]

The recent controversy[7,8] regarding the necessity for fats in the diet fails to emphasize adequately the distinction in the significance of fats as sources of energy, carriers of vitamine, and of lipoids regarding the rôle of which we are still largely uninformed.

After citing the ability of rats to grow on a diet of casein, wheat bran, wheat starch, and butter in contrast with the failure to thrive in the absence of the butter, Aron[7] has insisted that food fats have a specific nutrient value, "Sondernährwert," which cannot be replaced by other foodstuffs. Thus he says: "The special nutritive value of food fats probably involves the presence of certain lipoids."[9] Aron's thesis is essentially another plea for the recognition in some natural fats of nutritive factors not expressed in terms of calories alone.

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 311.

[5] Osborne and Mendel,[2] p. 84.

[6] A discussion of this problem is being prepared for publication.

[7] Aron, H., *Biochem. Z.*, 1918, xcii, 211.

[8] Aron, H., *Biochem. Z.*, 1920, ciii, 172. Salkowski, E., *Biochem. Z.*, 1919, xciv, 205. von Gröer, F., *Biochem. Z.*, 1919, xcvii, 311.

[9] Aron's actual experiments are not entirely convincing in so far as the benefits of the butter are ascribed entirely to the *fat* component. Butter carries a not negligible portion of non-fat milk solids which may include water-soluble vitamine, etc., in which his basal diet may have been low.

This by no means answers the fundamental question as to whether true fat is indispensable in the diet. Among the various explanations of the genesis of so called war edema has been the shortage of fats and of vitamines in the diet. Thus Schittenhelm and Schlecht[10] conclude from their extensive observations:

"Die Erfahrungen, welche wir heute über den Einfluss fettfreier Nahrung haben, beschränken sich völlig auf Tierexperimente. Die Tiere sind in ihrem Wachstum geschädigt und gehen schliesslich zugrunde. Besonders hervorstechende Krankheitserscheinungen scheinen dabei nicht aufzutreten. Wie sich die Wirkung andauernder fettfreier Ernährung beim Menschen äussern würde, müsste erst erwiesen werden. Man kann aber wohl annehmen, dass die Lipoidverarmung des Körpers zu Störungen Veranlassung geben kann, wenn man die lebenswichtige Funktion in Betracht zieht, welche den Lipoiden als mutmasslichen Bestandteil der Plasmahaut für die Aufnahme der Stoffe in die Zelle zukommt."

The experimental studies of Kohman[11] tend to make the fat deficiency explanation of nutritional edema untenable since they show that even in the presence of fats in the diet "a combination of low calories, low protein and excessive fluid intake will lead to a marked dropsy corresponding to war dropsy in all respects. The importance of specific vitamines seems to be excluded by these experiments."[12]

Maignon[13] supposes that the fats play an important rôle in the utilization of protein, a rôle which carbohydrates are powerless to fill. His experiments, conducted by feeding mixtures of more or less isolated food materials without due consideration to the now well recognized need of accessory food factors or vitamines, need not be further reviewed at this time.

Attention has recently been concentrated anew on this question by a publication of von Gröer[8] from Pirquet's clinic in Vienna. He has succeeded in attaining practically normal increments of weight in two infants who were fed during their first half year of life on a diet practically devoid of fat. This consisted of

[10] Schittenhelm, A., and Schlecht, H., Die Ödemkrankheit, Berlin, 1919; also *Z. ges. exp. Med.*, 1919, ix, 1.

[11] Kohman, E. A., *Am. J. Physiol.*, 1920, li, 378.

[12] Maver, M. B., *J. Am. Med. Assn.*, 1920, lxxiv, 934.

[13] Maignon, F., Recherches sur le rôle des graisses dans l'utilisation des albuminoïdes, Lyon, 1919.

highly centrifugated milk[14] supplemented with an energy equivalent of cane sugar. Later a porridge containing skimmed milk and cane sugar and having twice the fuel value of whole milk was fed. According to a personal communication from Professor von Gröer to one of us (M.) this diet was adhered to strictly. He concludes that "the theoretical fat minimum of the human suckling as shown by these two nutrition experiments is in any event of such a small magnitude that, contrary to the current views, it may practically be disregarded."

It might appear as if this apparent success contravened the experience of numerous investigators, including ourselves, regarding the indispensability of the fat-soluble vitamine for the normal nutrition of growing individuals.[15] Several considerations, however, must be taken into account. First, centrifugated milk is not *entirely* devoid of fat or of the fat-soluble vitamine.[16] We[17] have seen young rats grow to considerable size on diets of isolated foods in which the added daily supply of fat-soluble vitamine consisted of only 0.1 gm. of butter fat, and our early seemingly successful experiments with fat-free foods have already been alluded to. Second, it is by no means certain yet whether or not young animals have a certain store of this food factor which only gradually becomes exhausted.[18] Third, the period of growth in von Gröer's two clinical observations was comparatively short. 6

[14] Fat content about 0.01 per cent.

[15] Mendel, L. B., *New York State J. Med.*, 1920, xx, 212.

[16] *Cf.* McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 233.

[17] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549.

[18] The question is discussed in some detail in a paper by Osborne and Mendel, soon to appear; see also *Med. Research Comm., Nat. Health Insurance, Special Rep. Series, No. 38*, London, 1919, 16, which states:". . . . for a short time the young animals are able to grow when the fat-soluble factor is deficient in their diet. The explanation of this is probably supplied by the assumption that the animal organism normally contains reserve supplies o the factor 'A' in its own body. If this hypothes's is correct it s reasonable to assume that these reserves are mobilized for use when a deficiency occurs in the diet, but as soon as they are exhausted growth is immediately inhibited. During the period of temporary growth, throughout which it has been assumed that the reserve supplies are being utilized, the animals show a normal appearance and there does not appear to be any decline in their health, but when the reserves are exhausted and the deficiency becomes felt, not only do they cease to grow but they become highly susceptible to bacterial infection." See also Mendel.[15]

months of human growth corresponds to a few days only of rat growth. In von Gröer's experiments both children showed signs of interrupted growth in the course of the feeding periods (one of them after 3 months) and were attacked by illness which he ascribes to intercurrent infection during a prevailing epidemic of grip. The suspicion is raised that a lowered nutritive condition may have contributed to this interruption of growth—a conclusion in harmony with experiments on animals living on diets poor in fat-soluble vitamine.

In discussing von Gröer's experiments Aron[8] writes: "Young sucklings can develop apparently unharmed for several months on a diet practically fat-free; subsequently, however, disturbances in their well being arise."

He thereupon adds: "Children nourished on a diet poor in fat, as has been demonstrated on other occasions,[19] are not very resistant toward infections and are affected by them with exceptional severity."

Here again there is a confusion between the possible effects of a lack of fats *per se* and a deficiency of the vitamine factor which is associated with them in milk fat and elsewhere.

The ideal plan for ascertaining experimentally whether true fats are dispensable in nutrition would consist in adding the isolated fat-soluble vitamine to an absolutely fat-free otherwise adequate diet as the ration of growing animals. We have "concentrated" the fat-soluble vitamine by preparing "butter oil"[20] as well as by extracting potent fractions of "oils" from certain plant products.[17, 21]

The appreciation of the distribution of the fat-soluble vitamine in considerable concentration among plant products, notably green leaves, not ordinarily classed as fat foods has made it possible to approach a reasonably successful method of investigation. Consequently we have conducted new feeding experiments on young rats with diets presumably containing adequate amounts of both the fat-soluble and water-soluble vitamines, but exceedingly poor in true fats; *i.e.*, compounds soluble in pure

[19] Niemann, *Berl. klin. Woch.*, 1919, 777.

[20] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 379.

[21] Osborne, T. B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1918–19, xvi, 98. Zilva, S. S., *Biochem. J.*, 1920, xiv, 494.

ether. The fat-soluble factor was supplied by dried alfalfa,[22] the water-soluble by dried yeast. The foods were prepared as follows.[23]

The starch was first mixed with cold water and then gelatinized by pouring into boiling water. The meat residue and salts were added and the mixture was dried in air at about 70°.

In the first series (Rats 6369, 6367, 6383) the food mixture consisted of meat residue 23, starch 72, and salt mixture 5 per cent. To furnish the necessary vitamines tablets containing 0.4 gm. of brewery yeast, 0.2 gm. of alfalfa, and 0.2 gm. of cane sugar were fed daily apart from the rest of the food which was offered *ad libitum*. Rats 6367 and 6383 received additional doses of alfalfa, as indicated on Chart 1, during the latter period of their growth.

The meat residue[24] yielded 1.6 per cent of ether extract. The diet can at best be described as exceptionally low in true fats. Nevertheless the animals grew on it with vigor, reaching approximately 300 gm. when this report was prepared. In two cases (Rats 6367, 6383) the daily supply of alfalfa was increased to 1 and 0.4 gm. respectively at a time when it seemed as if the amount of fat-soluble vitamine might be too small. This change increased very slightly the amount of true fat available for the animals.

In order to reduce still further the fat component of the diet which, except in the very small fixed daily portions of alfalfa and yeast, increased with the augmented food intake of the large animals, a food mixture was prepared for which the thoroughly dried meat residue was extracted five times with ether containing a little alcohol.

Extracting with anhydrous ether removed from the non-vitamine portion of the food mixture 0.24 per cent of solids. On the basis of these data the largest daily intake of ether extract (fat(?))

[22] The preparation and potency of this from young alfalfa leaves is described by Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187. Its content of ether-soluble material was 5.06 per cent. 0.2 gm. of this dried alfalfa, as supplied daily to the rats, would thus contain only 0.01 gm. of fat (ether extract).

[23] The general conduct of such experiments by us has been described by Ferry, E. L., *J. Lab. and Clin. Med.*, 1920, v, 735.

[24] For the preparation of this, see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 369.

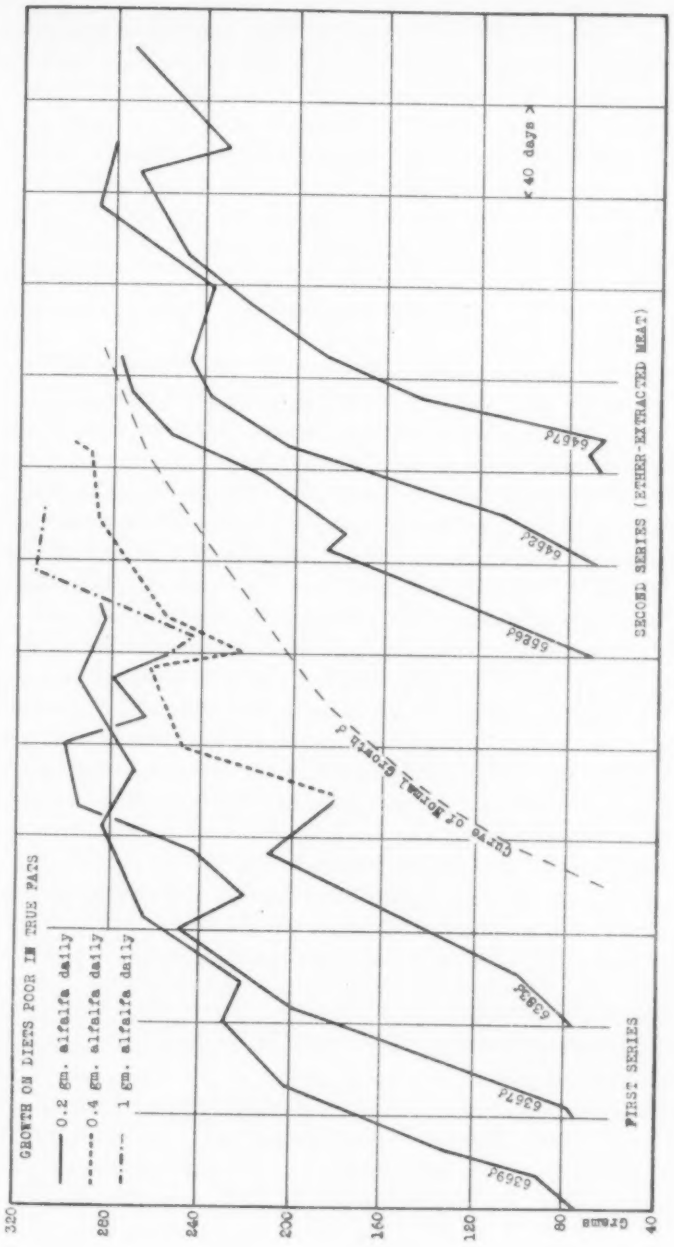
GROWTH ON DIETS POOR IN TRUE FATS
0.2 gm. alfalfa daily
0.4 gm. alfalfa daily
1 gm. alfalfa daily
CURVE OF NORMAL GROWTH
FIRST SERIES
SECOND SERIES (ETHER-EXTRACTED MEAT)
40 days
Grams
320
280
240
200
160
120
80
40
63694
63674
63834
64674
64664
65864

CHART 1.

for any of the animals on this series was 0.078 gm., and this during a period when the weekly food intake reached the exceptional amount of 174 gm. These rats (Nos. 6526, 6452, 6457), like those of the first series, have shown growth to large size (Chart 1). Inasmuch as all the animals starting on the diet with a body weight of approximately 70 gm. have quadrupled their weight within the usual time, and appear as well nourished as companion rats on diets containing liberal portions of butter fat or lard, we cannot avoid the conclusion that *if true fats are essential for nutrition during growth the minimum necessary must be exceedingly small.*

Hindhede,[25] whose results with young men correspond to observations on laboratory animals, has contended that fat is not required in the diet if a sufficient amount of fresh fruits and vegetables is eaten daily to supply vitamines.

The 80 to 90 gm. of the fat-free diets (about 3.8 calories per gm.) voluntarily eaten by the rats compared with the 60 to 70 gm. of our standard experimental diets containing 28 per cent of fat (about $5\frac{1}{2}$ calories per gm.) usually eaten by rats of similar size represents the adjustment to the energy needs of the animals.

As they grew larger the difference in the consumption of the "fat-free" over the "standard" ration became even more striking. Thus at a body weight of 270 gm. the animals frequently ate 125 to 160 gm. or more of fat-free food per week in contrast with an average of 82 gm. on the standard diet. Incidentally the large intakes have resulted in the ingestion of large quantities of protein. This has evidently not been detrimental to the rate of growth of the animals. On the other hand, in view of the very rapid gains made by these animals during the early weeks of the experiments, it may even be debated whether it has not represented an actual advantage in the nutrition of growth.

[25] Hindhede, M., *Molkerei-Ztg.*, 1918, xxviii, 152; abstracted in *Chem. Zentr.*, 1918, ii, 745; *Skand. Arch. Physiol.*, 1920, xxxix, 78.

THE PHYSIOLOGICAL EFFECTS OF SHORT EXPOSURES TO LOW PRESSURE.

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Although it has been recognized that prolonged sojourns at high elevations tend to upset the physiological equilibria and result in a permanent stimulation of the hematopoietic apparatus, the time required for these changes still remains a matter of conjecture. In regard to exposures to low pressure of short duration such as are met with in aeroplane flights, the current opinion is that the mainly respiratory and circulatory effects are of a transitory character, and that the cellular life of the body is left undisturbed. If it could be demonstrated that a few hours stay in high altitudes is sufficient to stimulate the blood-forming organs to increased activity, such a discovery would be, from a therapeutical point of view and taking into consideration the ease in reaching high elevation that is offered by modern aviation, of a far reaching interest. It must be expected, however, considering the difficulties that for a long time kept the blood changes during prolonged sojourns at high elevations open to argument, that the crucial test of a stimulation of the bone marrow occurring from only a short exposure to low pressure would be still more difficult to produce.

The older data on the subject, obtained from observations during balloon ascensions, are contradictory. While Gaule (1) claimed that he found evidence of a stimulated formation of red cells, von Schroetter and Zuntz (2) were unable to confirm his observations; they admit, however, that the blood-forming organs may be hyperemic after a few hours stay at an altitude of 4,000 meters. The permanent increase of erythrocytes Kuhn (3) and others have observed from use of the "suction mask" has been attributed by them to a direct effect on the bone mar-

row, and, although it must remain doubtful whether their procedure can be compared with an exposure to low pressure, their extended time of observation seems to warrant their conclusion that the bone marrow may be sensitive to a deficient oxygen supply of very short duration.

Gregg, Lutz, and Schneider (4) in a recent piece of research, in which they interned a great number of men in a low pressure chamber for short periods of time, found in the majority of cases an increase in the number of red cells, but they are inclined to the view that an "increased production of hemoglobin and erythrocytes by the bone marrow is improbable" and that the change is due either to a concentration of blood plasma or to a dormant supply of old blood corpuscles.

The emphasis Abderhalden (5) has laid upon the necessity of combining blood countings with biochemical methods for reaching an understanding regarding the blood changes during prolonged sojourns in high altitudes, seems to hold true also in the controversy whether the increase of red cells, occasionally observed after short exposures to low pressure, is caused by a stimulation of the erythropoietic organs or not. It is evident that the biochemical methods one of us (6) has employed to test the increase of erythrocytes in mountain trips would be useless for our present problem; that, however, the biochemical equilibria of the body may be profoundly affected by short exposures to low pressure may be learned from investigations of Haldane and coworkers (7) in which they found changes of the acid-base equilibria, similar to those that have been demonstrated by one of us (8) to take place during prolonged stay at high elevation.

Masing (9) has suggested the use of the phosphorus content of the erythrocytes as a criterion of the presence of newly formed red cells; a higher percentage would then indicate a stimulation of the erythropoietic organs. Considering the presence, according to the hematologists, of nuclear remnants in the circulating red cells, Masing's idea seems to be worthy of adoption, in the light of recent investigations of Bloor (10). It is, however, doubtful whether any decision can be reached as to the average age of the erythrocytes from analysis of the red corpuscles for phosphoric acid. Furthermore as it has been found that the corpuscle volume may vary within wide limits, the rough figure

for the phosphorus content of a unit volume of packed corpuscles is misleading for our present purpose. The average phosphorus content of an individual red cell, which for our inquiry would be instructive, is affected with such an excessive experimental error as in most cases to make its use imaginary.

Recognizing the obstacles referred to above, but anyhow hoping to arrive at results that might throw some light on the question whether any stimulation of the blood-forming organs occurs when they are subjected to a decreased oxygen supply, we decided to combine estimations of the phosphorus content of the blood with blood countings in animals kept for a few hours at artificially decreased pressure. We were the more induced to undertake the investigation as no data are available regarding the phosphorus content of the blood in high altitudes; and in addition as such a research would furnish a great number of data with reference to the normal variations of the total phosphoric acid and its different fractions. One of us (6) has speculated whether the constant phosphorus retention he found, combined with a lowered bicarbonate content of the blood, could possibly depend on the participation to a greater extent of the phosphates in the buffer value of the blood in high altitudes. It may be said in this connection that our present investigation wholly disproves this suggestion, which was formulated because of lack of information of the high phosphorus content of the red cells, which fully explains the positive phosphorus balances observed.

We employed for our experiments a low pressure chamber that had been constructed by one of us (E. S. S.) for artificial high altitude experiments on small animals. The apparatus consists of a cylindrical vessel made of heavy galvanized sheet iron (height 50, diameter 65 cm.) with concave bottom and top, and with twelve vertical wrought iron braces branching off to support the top. In the center of this is a round window, 35 cm. in diameter, surrounded by a rim for a rubber gasket. On the rubber gasket is placed a piece of rubber tubing on which rests a piece of plate glass, $\frac{5}{8}$ inch thick. By this simple arrangement a hermetical sealing is accomplished when the pressure inside the apparatus is decreased. The evacuation of air is done with a suction pump, driven by a $\frac{1}{4}$ horse power electrical motor. A constant pressure is attained by adding to the constant air inlet an intermittent one, which is controlled by a mercury manometer.

When the pressure reaches a certain point a contact is made, closing a circuit from two dry cells, which in turn by means of a relay closes the main circuit and opens a valve in a modified gas faucet, connecting the chamber for a moment with the outside air. This automatic device can be relied upon to keep the pressure inside the apparatus constant for any length of time, even at a vacuum of 650 mm. For experiments extending over longer periods of time the apparatus is supplied with a device for feeding the animal, with an arrrangement to collect urine and feces, which consists of an inner enameled metal funnel around the wall to half its height and passing through the bottom of the chamber. The feces are collected on a wire net suspended above the bottom of the funnel. A water-sprinkling device of simple construction is further added in order to rinse off the urine at the end of certain periods without necessitating the lowering of pressure.

As experimental animals rabbits were chosen, as their quiet habits minimize the errors that might in a work of this kind result from excessive struggling The blood samples were drawn from the heart, one before putting the animal into the apparatus, the other immediately after restoring normal pressure. To enable quicker handling the animal was tied down on its back in a box in as comfortable a position as possible and it soon became quiet, especially when the pressure went down. While thus in the low pressure rabbits were comparatively quiet; the control animals, which were also tied down and otherwise treated exactly as the former ones, occasionally tried to pull themselves loose. The experiments were started in the morning before the rabbits had been fed. The reduction of pressure was rapid, requiring only 2 to 3 minutes, and the restoration of normal pressure took place in about the same length of time.

In the first ten experiments 25 cc. of blood were drawn, in other experiments only 15 cc. were taken. 0.5 per cent of powdered potassium oxalate was added to prevent clotting. The same hemocytometer was used throughout the work and only duplicate countings that were nearly identical were accepted.

In order to test out whether the peripheral blood differs in its red count from the blood from central organs we did a few parallel countings in the blood from the heart and from the ear vein as well. The figures agreed with each other within the limits of experimental error.

The methods for the determination of phosphoric acid constituents in blood have been described previously (11) and certain modifications of the methods which have been used in this work are discussed in the following paper (10). A special form of hematocrit was used for the work and since it has proved accurate and satisfactory a brief description is desirable. The apparatus consists of the hematocrit proper and a container. In making the hematocrit two sections of capillary tubing of even bore, 1 mm. inside, 5 mm. outside diameter, and 150 mm. long, were cut off and the sharp edges of the ends beveled a little so that they would not break when used in the centrifuge. The container consists of a length of glass tubing about 120 mm. long, 15 mm. inside diameter, and with walls at least 1 mm. thick. This was fitted with a 10 mm. section of solid rubber stopper at one end and with a stopper perforated to admit the hematocrit tube at the other.

In use the well mixed blood is drawn up into the hematocrit tube to a height of 12 or 13 cm., held there by a finger over the upper end, the excess blood wiped off, and the lower end sealed by a disk of gummed label cut to the size of the tube. The hematocrit containing the blood is then placed in the container through the perforated stopper at the top, pushed firmly against the stopper in the bottom, and the container placed in the metal tube of the centrifuge. After centrifugation the length of the column of total blood and of corpuscles is measured with an accurate scale and the percentage of corpuscles calculated. The variation in values of duplicate samples using these tubes is generally within 1 per cent of the total value. Values obtained with the hematocrit are always lower, generally one or two points, than values obtained from ordinary graduated centrifuge tubes under the same conditions.

Of thirty-one experiments in all, nineteen were low pressure experiments. In three experiments the pressure was reduced to to 350 mm., in eleven to 400, and in five to 450 mm. Twelve were control experiments at normal pressure. One attempt was made to bring the pressure down to 300 mm., but the rabbit died after $\frac{1}{2}$ hour. In twenty-one experiments the animals were exposed to low pressure 3 hours, in the rest the time varied from $1\frac{1}{2}$ to $5\frac{1}{2}$ hours.

TABLE I.

Red Corpuscles, Hematocrit Value, Corpuscle Volume, and Phosphoric Acid Content—Total, Inorganic, Organic, and Lipoid—in Corpuscles and Plasma per 100 Cc.

								Phosphoric acid in corpuscles.				Phosphoric acid in plasma.			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
No. of experiment.	Weight.	Pressure.	Time.	Series.	No. of red corpuscles.	Hematocrit value.	Corpuscle volume.	Total.	Inorganic.	Organic.	Lipoid	Total.	Inorganic	Organic.	Lipoid.
	gm.	*mm.*	*hrs.*		*millions*		μ^3	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	4,230	400	4	A	6.09	44.8	73.6	289	20	231	38	21.4	10.3	0.4	10.4
				B	6.52	40.7	62.4	324	13	268	37	16.5	8.3	0	7.5
2	4,425	400	3½	A	6.03	34.2	56.7	283	19	205	68	23.5	8.3	1.9	16.5
				B	6.33	37.3	58.9	283	14	200	60	19.0			
3	3,500	350	2	A	5.32	33.1	62.2	400	9	309	70	21.0	11.3		9.4
				B	5.91	30.0	50.8	442	7	339	80	18.0	9.0		8.7
4	4,430	400	2½	A	6.96	40.5	58.2	338	14	266	63	26.0	11.9		13.9
				B	6.42	39.3	61.2	359	13	277	68	21.1	10.0		11.5
5	3,500	350	1½	A	6.23	42.2	67.7	350	15	256	60	24.1	10.2	3.0	11.5
				B	6.09	40.0	65.7	355	14	267	52	20.0	8.5	2.5	8.6
6	3,100	350	2	A	7.19	39.7	55.2	414				24.0			
				B	6.20	29.0	46.8	470				17.3			
7	4,425	400	5½	A	5.67	34.0	60.0	333		240	82	34.0	9.7	1.6	25.0
				B	5.29	35.2	66.5	351		252	82	36.0	10.8	1.1	25.0
8	4,425	400	2½	A	6.62	41.2	62.2	342		293	65	27.0	11.1	6.2	8.7
				B	6.45	36.4	56.4	393		310	68	20.0	10.3	3.8	5.2
9	3,000	400	1½	A	5.55	34.9	62.9	350	9.0			19.3	10.3	4.0	5.0
				B	5.90	37.7	63.9	350	8.3		62	15.0	7.8	2.2	5.0
10	3,100	400	2	A	6.54	37.6	57.5	358				21.1			
				B	6.91	37.1	53.7	342				16.1			
11	4,400	450	3	A	6.21	35.1	56.5	350			66	20.0	9.5		11.5
				B	5.36	33.2	62.0	350			64	14.0	4.8		9.7

TABLE I—*Continued.*

								Phosphoric acid in corpuscles.				Phosphoric acid in plasma.			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
No. of experiment.	Weight.	Pressure.	Time.	Series.	No. of red corpuscles	Hematocrit value.	Corpuscle volume.	Total.	Inorganic.	Organic.	Lipoid.	Total.	Inorganic.	Organic.	Lipoid.
	gm.	*mm.*	*hrs.*		*millions*		μ^3	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
12	3,375	450	3	A	6.35	32.5	51.1	350			57	32.0	10.0		20.0
				B	5.43	30.6	56.4	350			57	23.0	6.6		17.0
13	4,340	450	3	A	4.68	30.0	64.1	425			70	29.5	11.4		17.5
				B	4.34	28.6	65.9	395			70	22.0	7.5		15.0
14	3,360	450	3	A	5.49	35.3	64.3	343			66	30.0	14.0		12.0
				B	5.39	30.5	56.6	365			72	24.3	11.0		10.0
15	3,300	450	3	A	6.27	32.8	52.3	455	405*		69	23.0	10.0		11.5
				B	6.44	31.3	48.6	460	400*		68	24.0	11.8		10.0
16	4,100	400	3	A	4.92	30.2	61.4	401	300*		98	27.4	10.5		17.0
				B	4.34	28.3	65.2	450	360*		98	24.2	6.8		15.0
17	3,580	400	3	A	5.27	36.3	68.9	330	10	270		17.5	6.8		11.3
				B	5.22	34.7	60.7	352	10			16.3	6.0		10.4
18	3,640	400	3	A	4.84	29.5	61.0	345	28	280	67	18.4	6.7		11.1
				B	4.47	26.2	58.6	340	25	290	60	14.0	4.5		9.1
19	3,950	400	3	A	4.91	30.4	61.9	350		270	70	22.0	9.4		11.5
				B	5.14	29.4	57.2	378		280	75	14.0	5.0		9.5
20	4,100	760	3	A	5.28	29.8	56.4	385	18.9	265	85	24.2	11.5	1.0	10.1
	Same as No. 16.			B	5.86	30.0	51.2	390	20.6	264	92	19.7	7.5	2.5	12.0
21	3,580	760	3	A	5.54	36.6	66.0	325	26.0	230	72	21.7	6.7	2.3	11.1
	Same as No. 17.			B	5.88	33.3	56.6	320	26.0	224	75	21.0	7.5	1.5	10.1
22	3,070	760	3	A	5.46	32.2	59.0	320	6.0	219	63	26.2	12.2		10.7
				B	5.12	31.9	62.3	320	11.1	214	66	26.2	10.0		10.0

* Acid-soluble.

TABLE I—*Concluded.*

								Phosphoric acid in corpuscles.				Phosphoric acid in plasma.			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
No. of experiment.	Weight.	Pressure.	Time.	Series.	No. of red corpuscles	Hematocrit value.	Corpuscle volume.	Total.	Inorganic.	Organic.	Lipoid.	Total.	Inorganic.	Organic	Lipoid.
	gm.	*mm.*	*hrs.*		*millions*		μ^3	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
23	3,950	760	3	A	5.02	30.4	60.6	391	10.7	274	67	28.6	11.2	2.8	13.5
	Same as No. 19.			B	5.06	30.0	59.3	390	10.7	275	65	23.3	9.2	2.6	13.5
24	2,780	760	3	A	5.07	27.5	54.2	336	20.0	236	85	22.5	9.1	1.9	11.0
				B	3.90	24.7	63.3	330	22.0	233	90	16.0	5.0	2.5	8.5
25	3,040	760	3	A	3.34	21.7	65.0	470	20.0	315	110	27.4	10.5	5.5	10.0
				B	3.86	26.0	67.4	490	16.0	339	120	30.0	12.0	2.0	14.3
26	3,830	760	3	A	5.00	31.9	63.8	500	8.3	292	65	27.4	7.5	2.8	13.0
				B	4.90	30.5	62.2	445	10.0	310	90	27.4	7.5	2.5	13.0
27	4,260	760	3	A	5.47	34.9	63.8	330	±	255	70	21.7	10.0	3.4	8.9
				B	5.71	37.9	66.4	330	±	255	75	17.0	5.9	2.2	8.8
28	3,460	760	3	A	5.75	36.4	63.3	280	8.0	232	50	21.5	10.0	2.5	10.0
				B	5.51	34.6	62.8	317	11.5	264	48	19.0	6.4	3.0	9.4
29	3,150	760	3	A	5.81	38.0	65.4	300	14.0	251	53	26.2	12.0	4.0	10.2
				B	5.51	33.3	60.4	325	15.6	254	53	24.2	11.0	3.0	10.2
30	3,440	760	3	A	4.97	33.3	67.2	250	8.0	200	53	19.5	5.0	5.5	9.5
				B	4.46	30.8	69.1	250	8.8	202	53	18.5	4.0	5.0	9.7
31	2,880	760	3	A	6.34	35.0	55.2	360	19.0	273	65		6.2	2.8	12.2
				B	6.03	32.9	54.6	350	16.0	259	63		6.0	3.8	12.2

In Table I the results of the experiments are recorded respectively for red count, hematocrit value, corpuscle volume (obtained by dividing the volume of red corpuscles in 1 c.mm. of blood as expressed by the hematocrit value in one-thousand millionth of a c.mm., or as we prefer to express it in μ^3 as unit, by the number of red cells in 1 c.mm. of blood), and phosphoric acid content in blood

corpuscles and plasma, both the directly determined total amount and its fractions—inorganic, organic, and lipoid. In the table, A designates the series at the beginning and B the series at the end of each experiment.

The complete analysis of the blood of twenty-eight normal rabbits for phosphoric acid constituents as given in Table I makes it possible to establish tentative average values and variations for these constituents in normal rabbits.

Blood Count.—Thirty samples (including extra samples at different times from Rabbits 16, 17, and 19 and excluding No. 25 which was definitely anemic). Average 5.66; highest 7.19; lowest 4.68 millions.

Hematocrit Value.—On the same animals. Average 34.67; highest 48; lowest 27.5 per cent.

Plasma Phosphate.—Inorganic Phosphate.—Twenty-six animals. Average 9.76; highest 14; lowest 5.0 mg. per 100 cc.

Lipoid Phosphate.—Twenty-five animals. Average 11.46; highest 25; lowest 5.0 mg.

Corpuscle Phosphate.—Inorganic Phosphate.—Sixteen animals. Average 14.2; highest 28; lowest less than 3.0 mg. per 100 cc. of corpuscles.

Organic Phosphate.—Nineteen animals. Average 257; highest 315 (in the anemic rabbit); lowest 200 mg. per 100 cc.

Lipoid Phosphate.—Average for 27 animals 68.4; highest 110; lowest 38 mg. per 100 cc.

Since for our present study the absolute figures are of minor importance, the interest centering around the changes taking place in the blood from the exposure to the low pressure, we have in Table II calculated the percentage changes of the number of erythrocytes, corpuscle volume, corpuscle phosphates, and plasma phosphates, total, inorganic, and lipoid. The experiments are arranged according to pressure and time of exposure.

It is regrettable that our experimental methods involved a loss of 5 to 6 per cent of the blood of the rabbit at the commencement of each experiment. It is well known that large hemorrhages themselves may stimulate the blood-forming organs. *A priori* it is impossible to tell to what extent the hemorrhage might have interfered with the effect of the internment in the low pressure chamber. On the other side the objection may be raised that the awkward position of the animal during the experiment in some way may affect the composition of the blood. As already mentioned real struggling of the animal very seldom

TABLE II.

Changes in the Number of Red Corpuscles, Corpuscle Volume, Total Phosphorus of Red Cells, and Total, Inorganic, and Lipoid Phosphorus in Plasma.

No. of experiment.	Pressure.	Time.	No. of red cells.	Corpuscle volume.	Phosphorus.			
					Total in corpuscles.	Total in plasma.	Inorganic in plasma.	Lipoid in plasma.
	mm.	*hrs.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
"Ideal" controls.								
22	760	2½	−6.2	+4.2	0	0	−18.0	−6.5
26	760	3	−2.0	−2.5	−11.0	0	0	0
27	760	3	+4.4	+4.1	0	−21.7	−41.0	−1.1
28	760	3	−4.2	−0.8	+4.5	−11.6	−36.0	−6.0
29	760	3	−5.2	−7.6	+8.5	−7.6	−8.3	0
30	760	3	−10.3	+2.8	−3.8	−5.1	−20.0	+2.1
31	760	3	−4.9	−1.1	−5.1	+1.0	−3.2	0
Controls, second exposure.								
20	760	3	+11.0	−9.2	+1.3	−18.6	−34.8	+18.8
21	760	3	+6.1	−15.2	−1.5	−3.2	+11.9	−9.0
22	760	3	+0.8	−2.1	−0.2	−18.5	−17.9	0
Control.								
25*	760	3	+15.6	+3.7	+4.3	+9.5	+14.3	+43.0
Control.								
24†	760	3	(−22.9)	(+16.8)	−1.8	−28.9	−45.1	−22.3
Low pressure experiments.								
5	350	1½	−2.2	−2.9	+1.4	−17.0	−16.7	−25.2
3	350	2	+11.1	−18.3	+10.5	−14.3	−20.4	−7.4
6	350	2	−13.8	−15.2	+13.5	−27.9		
7	400	1½	+6.3	+1.6	0	−22.3	−24.3	0
10	400	2	+5.7	−6.6	−4.5	−27.9		
4	400	2⅓	−7.8	+5.2	+6.2	−18.8	−16.0	−17.3
8	400	2⅓	−2.6	−9.3	+14.9	−25.9	+7.2	−3.8
16	400	3	−11.8	+6.2	+12.2	−11.7	−35.2	−11.8
17	400	3	+8.5	−11.9	+6.7	−6.9	−11.8	−8.0
18	400	3	−7.6	−3.9	−1.5	−23.9	−32.8	−18.0
19	400	3	+4.7	−7.6	+8.0	−36.4	−46.8	−17.3
2	400	3⅓	+5.0	+3.9	0	−19.1		
1	400	4	+7.1	−15.2	+12.1	−23.0	−19.4	−27.9
7	400	5½	−6.7	+10.8	+5.4	+5.9	+11.3	0
11	450	3	−13.7	+9.7	0	−30.0	−49.5	−15.7
12	450	3	−14.5	+10.4	0	−28.1	−34.0	−15.0
13	450	3	−7.3	+2.8	−7.0	−25.4	−34.2	−14.2
14	450	3	−1.8	−12.0	+7.0	−19.0	−21.4	−16.7
15	450	3	+2.7	−7.0	+1.1	+4.3	+18.0	−13.1

* Anemic; red corpuscles 3.34 millions.

† Very small rabbit, 2.78 kilos.

took place. By adding a great number of control experiments, in which the rabbits were treated in exactly the same manner as in the low pressure experiments, we believe that we have overcome the above named difficulties. As will be seen some changes occurred to about the same degree in both the main series of experiments but it would be difficult to understand why other changes were met with only in the low pressure series, if it were not that these changes are attributable only to the new environmental factors of the low pressure apparatus.

Of the twelve controls the first seven (in Table II) were in all respects ideal ones. In the next three the rabbits had 3 weeks previously been used for low pressure experiments and presumably because of this earlier "sensitizing" these experiments present differences from the other controls. In the next experiment the rabbit was anemic at the beginning and consequently may be supposed to have reacted more strongly to the hemorrhage than the others; while in the last of the controls the rabbit was unusually small.

Turning our attention to the percentage changes in the ideal controls we recognize that they are, with sole exception of the red count and inorganic phosphates, small and in most cases within the limits of experimental error. The decrease of the number of red corpuscles varies with exception of only No. 26 between 2.0 and 10.3 per cent, with an average of 5.5 per cent. When we calculate the blood volume from the body weight and compare the amount of drawn blood with this hypothetical blood volume, we arrive at approximately the same figure. It thus appears as if the decrease of red count bears a relation to the amount of hemorrhage, which seems to indicate that no dormant supply of erythrocytes was drawn upon.

The changes in the corpuscle volume vary between +4.2 and −7.6 per cent and as the experimental error of the corpuscle volume equals the superposed errors of two different estimations, we are inclined to the view that no change of the corpuscle volume took place in the ideal controls.

The total phosphorus content of the packed corpuscles varies in the seven experiments between +8.5 and −11.0 per cent. As the figures in five of the cases are well within experimental error, it is doubtful whether any significance can be attached to the

two somewhat larger deviations. Moreover, the problem, that for our present inquiry would be more important than the phosphorus content of the mass of mixed corpuscles, namely the average phosphorus content of an individual red cell, is difficult to approach because of the trebled experimental error. We have, however, satisfied ourselves as to this matter, but we have failed to discover anything that would point in the direction of a change of the phosphorus content of the single erythrocyte. The deviations were equally distributed around the average.

Regarding the change of phosphorus content of the plasma we notice a decided difference between the two main fractions, inorganic and lipoid phosphorus. While the change of the inorganic phosphates is, in all cases except one, strongly negative the lipoid fraction remains remarkably constant, the total phosphates occupying an intermediate position. The average decrease of inorganic phosphates is 18.1 per cent, of lipoids 1.6 per cent. It seems possible, therefore, that the lipoids were little if at all affected by the restoration of the blood to its normal volume.

Postponing the discussion of the remaining control experiments at normal pressure till later, we pass over to a consideration of the low pressure experiments. The number of red corpuscles (Table II) does not allow us to draw any sweeping conclusions, as the percentage changes vary between +11.1 and −14.5. Since the positive deviations occur mostly at the lower pressures, although the amount of blood drawn was in these cases somewhat larger, it is, however, probable that increase of red cells, notwithstanding the loss of blood, was due to the lowered atmospheric pressure. If 5 per cent of the negative changes is considered as due to the loss of blood we find that in the majority of cases at 350 and 400 mm. an increase of erythrocytes had taken place. But even the negative changes are noteworthy, as in high altitude researches it has often been found that a decreasing effect on the number of erythrocytes may precede the increasing one, making it probable that lability of the number of red corpuscles is a typical feature. On the other hand, although naturally a stimulation of the erythropoietic organs most frequently is accompanied by an increase of circulating red corpuscles, an absence of this latter phenomenon does not seem to exclude a

greater activity of the bone marrow, as it is possible that the newly formed red cells may linger there for some time before they enter the circulation.

The change of the corpuscle volume varies between +10.8 and −18.3 per cent. The maximal negative changes, which undoubtedly exceed the experimental error, are found after exposures to very low pressure. The largest positive change is reached after an exceptionally long exposure (5½ hours) to 400 mm. We are therefore inclined to the view that the present study confirms the results reported by one of us (12), that low pressure tends to alter the size of the erythrocytes. Whether these changes bear any relation to changes in the hydrogen ion concentration of the blood, as suggested, we are not in a position to answer, although the determination of the dissociation curve of the oxyhemoglobin in some of the experiments apparently pointed in this direction.

While very little weight can be laid upon averages, when the number of cases is small, it occurred to us that it might be of some interest to calculate the average change of corpuscle volume at different pressures.

Pressure. *mm.*	Corpuscle volume. *per cent*
350	−12.1
400	−3.8
450	+0.8
760	−0.8

The changes in the total phosphoric acid content of the corpuscles vary between +14.9 and −7.0 per cent. We further recognize that most of the changes are to the positive side and that the maximal ones of these occur at the lowest pressures. We would attach more importance to the prevalence of positive changes and believe that they indicate an increase of the phosphorus content of the corpuscles at high altitudes if we had succeeded in confirming them by calculating the phosphorus content of individual red cells. The maximal positive change is 19.1 per cent, and the maximal negative change is 11.1 per cent; the average change is +1.0 per cent. In the first seven control experiments the corresponding figures are 12.4, 13.2, and +1.2. In only two experiments is the positive change of the individual

corpuscle content of phosphorus safely beyond the limits of experimental error. In one of the cases the duration of the experiment was twice the average duration and it is possible that if we had extended the duration in other experiments still longer we would have been able to discover an increase of the individual corpuscle H_3PO_4 content proportional with the length of time.

The estimation of the lipoid phosphorus content in individual red cells revealed that only in three cases was the change beyond the limits of the experimental error. All these changes were negative (15.8, 17.0, and 13.9) and, as will be seen, coincided with maximal negative changes of the plasma lipoids.

If we turn our attention to the plasma phosphates in the low pressure experiments there seems to be no doubt that a decrease has taken place and this applies equally well to the inorganic and lipoid phosphorus and to the total phosphorus. The average decrease of the inorganic phosphorus is at 350 mm. 18.6, at 400 mm. 20.2, and at 450 mm. 24.2 per cent, with a total average of 21.3 per cent. The average decrease of the inorganic plasma phosphorus in the seven controls is 18.16 per cent. It therefore appears as if the decrease of the inorganic phosphates in plasma were attributable to the bleeding alone.

The decrease in the low pressure experiments for lipoid phosphorus is at 350 mm. 16.3, at 400 mm. 11.6, and at 450 mm. 14.9 per cent, with a total average of 13.2 per cent. When compared with the constancy of the lipoid phosphorus in the seven control experiments (average 1.6 per cent) these figures lead us to the conclusion that the decrease of lipoid phosphorus is caused by the low pressure treatment. The fact that no change of the lipoid phosphorus occurred in the two experiments of the shortest and longest duration seems to indicate that the greatest decrease takes place between $1\frac{1}{2}$ and $5\frac{1}{2}$ hours.

It now remains to seek an explanation for the decrease of plasma lipoids, which seems to be confined to the low pressure experiments. Effects of digestion and work are excluded as the animals were fasting and were tied down. Complete muscular inactivity could, naturally, not be obtained, but as to possible effects of struggling it has been pointed out that the control animals were more restless than the others. It seems certain that an enrichment of the circulating red cells with lipoids, that would

result in an impoverishment of the plasma in these substances, did not occur, as it was found that the greatest decreases of the plasma lipoids were accompanied by proportional decreases of the corpuscle lipoids. Thus the three maximal changes of plasma lipoids to the negative side (27.9, 25.2, and 18.2 per cent) corresponded to the three greatest changes in the same direction of the individual corpuscle lipoids (17.2, 15.8, and 13.9 per cent). Instead of compensating for the loss of lipoids from plasma by storing up lipoids, it is probable that even the circulating red cells may be compelled to contribute some of their own lipoids. But where are all these phospholipoids that probably consist mainly of lecithin deposited? While the inorganic phosphates are easily eliminated from the body through the kidneys (although such an explanation of their decrease in the plasma is doubtful in the light of the fact that a retention of phosphates is a constant phenomenon in high altitudes) such an output of the lipoids is out of the question. A dilution of the blood would likewise be contrary to the repeated observation that the blood plasma tends to become concentrated at high elevations. The most probable alternative is that the lipoids have been taken up by some part of the body itself.

A number of investigators have tried to elucidate the effect of small doses of lecithin on metabolism. In all the cases where the experiments have been well controlled (13) a retention of nitrogen and phosphorus and partly also of sulfur has been found, indicating a formation of new body protein. Other observers (14) have paid attention to the effect of injections of lecithin on the number of red corpuscles and have been able to find a rather constant increase. That lecithin probably plays some rôle in the function of the erythropoietic organs is evident from the fact that the bone marrow contains comparatively large amounts of lecithin and that this percentage is higher in the red marrow of young animals (15). Whether the hypothesis that the lecithin acts as a bridge to the nucleins, these proteins obtaining their phosphorus supply from the lipoids, will prove correct is impossible to tell; however, if that is the case, the beneficial effect of lecithin injections on the number of red cells would be satisfactorily explained. The lecithin requirements for the formation of new erythrocytes would, furthermore, throw light on the changes of the plasma

lipoids at low pressure. A part of these would be taken up by the blood-forming organs. If future investigations prove that this is true, the almost immediate reaction of the bone marrow to lowered pressure in the direction of a stimulation, which we are now able to offer only as a hypothesis, will at the same time obtain a firmer foundation.

As already mentioned, in the control experiments, Nos. 20, 21, and 23, the animals had 3 weeks previously been exposed to low pressure. One of us has directed attention to the sensitizing effect of sojourns in high altitudes (16) to very small changes in atmospheric pressure. It seems possible that the difference the three above named rabbits presented from the other controls can be explained in the same way; namely, that these animals reacted more strongly to the hemorrhage than the others. In spite of the loss of blood of all the rabbits, their blood contained a higher number of red cells after than before the experiment. The effect on the corpuscle volume was also more marked and in a negative direction. The effect on the plasma phosphates varied to a great extent; *e.g.*, in one animal the lipoid phosphorus increased by nearly 19 per cent. It deserves mentioning that the initial number of red corpuscles in the second experiment on the same animals exceeded the initial counting in the first series by 2.2 to 7.3 per cent. A greater number of comparative red counts would, however, be necessary to ascertain whether the stimulation of the bone marrow, that we have indicated, lasted for such a long period of time.

We have found that in normal rabbits a loss of about 5 per cent of the total blood volume does not seem to stimulate the bone marrow to greater activity during the first hours after the hemorrhage. It would have been interesting to study the effect of the bleeding of animals that at the onset of the experiment were anemic and of the effect of a somewhat proportionally larger hemorrhage. Only one experiment of each of these kinds is available in our series; namely, Experiments 25 (anemic rabbit) and 26 (smallest rabbit in all the series, therefore a proportionally greater loss of blood). In the former animal all the recorded blood changes are to the positive side. Expecially the increase of the number of red corpuscles and of the lipoid phosphorus of the plasma is noteworthy, but it would be futile in a single case

to speculate as to the coincidence of these two changes. In the latter experiment we are the more prevented from drawing any conclusions as the incongruence of the blood count and hematocrit value makes us suspect some error in the sampling of the blood for the counting. In such a case the figures for the red count and the corpuscle volume would be incorrect. However, the great decrease of all the main phosphorus fractions would then remain unexplained. It is possible that our estimations are correct and that the low body weight of the rabbit and therefore resulting disproportionally large loss of blood are responsible for the changes in question.

SUMMARY.

1. A self-regulating and inexpensive apparatus for animal experiments at low pressure is briefly described.

2. A convenient and accurate method for obtaining the hematocrit value by use of a high power centrifuge is given.

3. The variations of the content of phosphoric acid and its fractions—inorganic, organic, and lipoid—of the corpuscles and plasma of normal rabbits are discussed.

4. From comparisons of the percentage changes in the number of erythrocytes, of the corpuscle volume, and of the phosphorus content of corpuscles and plasma of rabbits that have been subjected to a small hemorrhage and then exposed for a short period of time on the one hand to normal pressure and on the other hand to pressures from 350 to 450 mm. it is concluded that:

(*a*) While at normal pressure the change of the number of red cells corresponds to the loss of blood from the experimental hemorrhage, the greater alteration of the number of erythrocytes at lower pressures is due to the low pressure.

(*b*) At very low pressures a decrease in the size of the red corpuscles occurs.

(*c*) The phosphorus content of the red cells does not undergo any marked change. In a few cases the decrease of the lipoid phosphorus is probably beyond experimental error.

(*d*) The change in the inorganic phosphorus of the plasma is negative, and of the same amplitude in control and low pressure series.

(*e*) The most characteristic feature of the low pressure experiments, when compared with the controls, is a constant decrease in the former of the lipoid phosphorus of the plasma.

5. It is suggested that the decrease of the lipoid phosphorus of the plasma at low pressure indicates an enrichment of the erythropoietic organs with lipoid material and that this may be the first phase of a stimulation of these organs resulting from a lowering of the atmospheric pressure.

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BLOOD PHOSPHATES IN THE LIPEMIA PRODUCED BY ACUTE EXPERIMENTAL ANEMIA IN RABBITS.

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It is probable that lecithin or a similar phospholipoid is an intermediate step in the utilization of fat in animals, since, aside from the close chemical relationship, it has been shown on the one hand that accumulation of fat in the liver in various conditions is probably followed by a transformation into phospholipoid in the organ (1), that during fat absorption lipoid phosphorus increases in the blood (2), and that in diabetic lipemia (3) the high values for fat are always accompanied by high lecithin values. On the other hand phospholipoid has been found to decrease in blood plasma during the formation of fat in milk secretion (4). In general it has been found that wherever there are variations in the fat of the blood they are accompanied by corresponding changes in the phospholipoid (3, 5).

In continuing the study of the relation of the lipoid phosphorus to fat metabolism, it seemed desirable to include a study of other phosphoric acid compounds in the blood so as to discover what relation, if any, existed between them and the lipoid phosphorus and what form of phosphoric acid combination participated in the formation of phospholipoid from fat. For the purpose of the study, the lipemia produced by acute experimental anemia described by Boggs and Morris (6) was chosen as a beginning, since this type of lipemia is easily produced in rabbits, reaches remarkable heights, and has been recently the subject of a study with regard to the variations in the blood lipoids during the course of the lipemia by Horiuchi (7). Moreover, as the result of some experimental work in this laboratory it seems likely that the condition is not easily produced in dogs, which offers a means

of differentiating between changes referable to the lipemia and those due to regeneration of blood corpuscles.

In the lipemia which follows repeated large bleedings in rabbits, Boggs and Morris found total fat values up to 4.5 per cent with fatty acids of high iodine value, and increase of lecithin, but no cholesterol. Horiuchi found similar high values for fat (total fatty acids) in plasma up to twenty-five times the normal value (4.4 per cent), increases of lecithin up to seven, and cholesterol up to eight times the normal values. The changes in the corpuscles were relatively much less, at no time rising much over twice the normal values for any of these constituents. Horiuchi found that the lipemia occurred whether the animal was on a high or low fat diet but was produced more readily and lasted longer on the high fat. In the work to be reported below a study has been made of the same condition from the point of view of the phosphoric acid compounds of the blood, both with the idea of inquiring into the relation of these compounds to the metabolism of fat and also of following the changes in these substances during the bleeding, in their relation to blood regeneration. It was desirable to find out for example which group of these compounds took part in the formation or resulted from the decomposition of lecithin and whether the organic phosphorus present in small amounts in plasma and in relatively very large amounts in corpuscles bore any relation to the loss of the nucleus in red cells during their formation.

A full discussion of the various phosphoric acid compounds of the blood was given in an earlier paper (8) but a short summary of the available knowledge on the subject will not be out of place here. In blood plasma, phosphoric acid is present as inorganic phosphate, as phospholipoid, and in small amounts as an unknown form soluble in acids; *i.e.*, organic phosphorus. In most samples of blood plasma the sum of the three—inorganic, lipoid, and organic phosphorus—is equal to the total phosphate within the limit of error of the determinations. In occasional samples another form of phosphoric acid combination is found which is insoluble in either acid ammonium sulfate or alcohol-ether, and which is probably nucleoprotein.

In blood corpuscles the same groups of compounds are found and again the sum of inorganic, lipoid, and organic is found to be

equal to the total phosphate in practically all normal samples examined. Nucleoprotein is therefore not normally present. In amounts these forms are generally much larger than in the plasma, the organic phosphorus being relatively very large and forming the main phosphoric acid constituent of the corpuscles. Corpuscle values tend to be much more constant than those of plasma and the determination of phosphates in whole blood is therefore not to be recommended when enough blood is available that plasma and corpuscles may be examined separately.

The methods used for the determination of the various phosphoric acid compounds have already been described in full (9), but certain modifications have been found desirable and are given below. For reasons noted above, plasma and corpuscles were analyzed separately, the separation being made in all cases by centrifugation at about 3,800 revolutions per minute for 10 minutes.

Total Phosphate.—1 cc. of plasma and 0.048 cc. of corpuscles were found to be the most suitable amounts for the determination in rabbit blood, in which the phosphoric acid content of the plasma is lower and that of the corpuscles higher than in human blood. In the digestion the second stage (boiling of the concentrated acid for about 8 minutes) has been omitted since it has been found that nothing is gained by the continued heating while there is a real danger of loss of phosphoric acid either mechanically or by vaporization. Digestion is carried only to the stage where the water is completely driven off, then if the mixture is colorless it is cooled, sugar added, and then the mixture is treated further as directed. If not colorless after the heatings, 1 or more drops of the reagent are added as required and the mixture is again heated. After the final heating the sulfuric acid solution should be clear and colorless. In occasional samples a yellowish tint persists in the hot solution, disappearing on cooling, which is impossible to remove, but since it does not appear to affect the accuracy of the results it has been neglected.

Standards Used.—For plasma 5 cc. of a standard containing 0.84 mg. of H_3PO_4 in 100 cc. of solution; for corpuscles 5 cc. of a standard containing 0.6 mg. of H_3PO_4 per 100 cc. (see note on standards below).

Lipoid Phosphate.—In normal rabbit plasma, alcohol-ether extract corresponding to 0.9 cc. of plasma was required. To obtain this amount 3 cc. of plasma were made up in 50 cc. of alcohol-ether as directed and 15 cc. used for the determination. As a standard 5 cc. of a solution containing 0.36 mg. of H_3PO_4 per 100 cc. were used. In many cases a weaker standard (0.24 mg.) is desirable.

In normal corpuscles the sample taken corresponded to 0.18 cc. of corpuscles in 10 cc. of alcohol-ether extract (see note below on corpuscle solutions). For a standard, 0.6 mg. of H_3PO_4 in 100 cc. was used.

Acid-Soluble Phosphate.—Total Acid-Soluble Phosphate.—For plasma 5 cc. of extract corresponding to 0.6 cc. of plasma have been found most suitable with a standard of 5 cc. of solution containing 0.36 mg. of H_3PO_4 per 100 cc. (properly adjusted as to salt content, etc.). In the digestion with H_2SO_4-HNO_3 the sugar treatment has been found unnecessary and has been omitted.

For corpuscles 2 cc. of extract corresponding to 0.0384 cc. of corpuscles have been used with a standard containing 0.36 mg. of H_3PO_4 per 100 cc. of solution.

Inorganic Phosphate.—For plasma 2 cc. of extract corresponding to 0.24 cc. of plasma were used with 2 cc. of a standard containing 1.2 mg. of H_3PO_4 per 100 cc.

Corpuscles.—The determination of inorganic phosphate in rabbit corpuscles has been found somewhat unsatisfactory. Values are widely variable and in one case (Rabbit B) protein was not completely removed by the acid ammonium sulfate. There is also uncertainty as to what extent the organic fraction is decomposed during the treatment, giving incorrectly higher values for inorganic phosphate. However, results are given of the determinations, which are at least of comparative value. Ordinarily 2 cc. of the extract, corresponding to 0.096 cc. of corpuscles, were used with 2 cc. of a standard containing 1.2 mg. of H_3PO_4 in 100 cc.

Standards.—Correction of the nephelometric readings has been found unsatisfactory and, in order to avoid the necessity of correction, a variety of standard solutions has been used. By selecting a standard solution not more than 30 per cent stronger or weaker than the solution to be measured the need for correc-

tion is avoided. The standards are conveniently made up in 100 cc. portions containing phosphate and salts as below. For total and lipoid phosphate in plasma and corpuscles, standards containing 0.84, 0.6, 0.36 mg. of H_3PO_4 in 100 cc. were used. In making these standards the phosphate solution is measured into a 100 cc. graduated flask, alkali equal to four times the amount used in neutralizing a single digestion mixture, ordinarily 18 cc., is added, then 4 drops of 0.3 per cent phenolphthalein are added, after which the solution is titrated to neutrality with the 1:1 sulfuric acid. In order to prevent the growth of mould about 12 drops more of the acid are added, the solution is cooled, and made to the mark. Moulds grow slowly in the neutral solution and soon remove most of the phosphate from it. Addition of excess acid as above has been found to prevent their growth in all but an occasional instance. If mould is detected the solution must be thrown out and a new standard made.

For acid-soluble phosphate in plasma, standards containing 0.24 and 0.36 mg. of phosphoric acid per 100 cc. were used. In making these solutions the phosphate is measured into a 100 cc. graduated flask, 8 drops of the phenolphthalein solution, 20 cc. of the alkali, and 18 cc. of the acid ammonium sulfate are added, and the solution is titrated to neutrality. 12 more drops of acid are added and the solution is cooled and made up to the mark. Moulds do not grow in this solution.

For acid-soluble phosphate in corpuscles, standards containing 0.36 and 0.6 mg. of H_3PO_4 were found to cover the range of values found. In preparing these standards the phosphate is measured into the graduated flask, 8 drops of phenolphthalein, 8 cc. of acid ammonium sulfate, and 18 cc. of the alkali are added, the solution is neutralized, and 12 drops of extra acid are added as before. The solution is then cooled and made up to the mark.

For inorganic phosphate in both corpuscles and plasma the standard as recommended (9) was found suitable except that, since 5 cc. of the standard and test solutions gave suspensions too concentrated for accurate reading, 2 cc. of each were used.

For these determinations and for nephelometric work in general, exactly parallel conditions in composition of solutions, time of standing after addition of reagent, temperature, amount of shaking, etc., must be preserved. It is desirable to establish a

routine procedure which should be as closely followed as conditions will permit.

Sampling of Corpuscles.—In experiments which involve extensive bleeding, as in those described below, where the percentage of corpuscles falls to a low level it is necessary to practice some economy so as to get enough material to complete the various analyses. The following scheme has been found satisfactory for rabbit blood: 3 cc. of the separated corpuscles are measured into a 10 cc. graduated flask, the pipette is rinsed out, and the washings and warm water are added to fill the flask to the mark. The whole is well shaken. After standing for at least 10 minutes for hemolysis to take place (in the ordinary routine from $\frac{1}{2}$ to 1 hour is allowed) and after again shaking, 4 cc. of the mixture are measured into acid ammonium sulfate in a 25 cc. flask for determinations of inorganic and acid-soluble phosphate. 3 cc. are measured into alcohol-ether for determination of lipoid phosphate and 2 cc. into water in a 25 cc. flask of which, after filling to the mark, 2 cc. are used for total phosphate.

Direct determinations were made of total, lipoid, inorganic, and acid-soluble phosphate. Organic phosphate is found by subtracting inorganic from acid-soluble.

The rabbits used in these experiments were on their ordinary diet of alfalfa and rolled barley which is a diet low in fat although containing more than the low fat diet used by Horiuchi. Blood samples were taken from the marginal ear vein, approximately 10 per cent of the blood volume (calculated as 8 per cent of the body weight) being taken daily. If the animal is previously exercised until the general circulation is stimulated and then the ear massaged a free and rapid flow of blood is obtained, yielding the desired amount in a few minutes. Clotting was prevented by the use of 1 per cent of saturated sodium citrate. The bleeding was continued daily until a satisfactory lipemia was established, then less frequently and taking only enough blood to follow the conditions during recovery. (About 15 cc. of blood were required to furnish material for a complete analysis.) Results are given in Table I. Six animals in all were studied of which complete reports are given of two while the others giving similar and less striking results are reported more briefly.

TABLE I.

Blood Phosphates in the Lipemia of Acute Experimental Anemia.

Day.	Blood taken.	Hematocrit.	Red count.	Corpuscle volume.	Phosphates per 100 cc.								Remarks.
					Plasma.				Corpuscles.				
					Total.	Inorganic.	Lipoid.	Organic.	Total.	Inorganic.	Lipoid.	Organic.	
	Rabbit A. Weight 8¼ lbs.												
	cc.	*per cent*	*millions*	*c.mm. × 10^{-8}*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
1	30	35.9	6.21	5.9	20.0	7.7	9.6	3.8	365	24	65	276	Plasma clear.
2	30	28.1	5.94	4.8		8.5	10.0	6.0	390	43	80	261	" "
3	30	21.5	5.23	4.2	28.5	11.0	11.5	3.0					" "
4	30	19.3	3.17	6.0	37.0	8.8	23.5	5.2	465	50	100	305	" moderately milky.
5	30	18.0	2.38	7.5	40.0	7.8	26.0	3.1	453				" "
6	30	16.9	1.68	10.0	45.0	10.0	26.0	4.0	562	62	140	332	Plasma milky.
7	30	16.5	2.07	8.0	47.0	11.0	30.5	5.0	532	88	156	287	" very milky.
8	15	16.1	1.87	8.6	50.0	17.6	30.0	2.4	560	95	155	280	" "
9	15	19.5	2.09	9.3	55.0	11.1	38.5	4.9	570	107	160	283	" "
10	15	20.0	2.53	8.0	48.0	9.4	37.5	2.1	555	83	162	297	Plasma milky.
11	15	21.0	2.93	7.2	48.0	11.0	33.3	3.4	555	50	113	301	" clear.
12	15	23.5	2.48	7.6	37.0	11.5	25.0	0.5	490	90	140	260	" "
13	15	23.6	3.04	7.8		9.0	17.2	5.0	550	50	130	340	" "
15	15	26.5	3.64	7.3	24.2	11.1	12.5	3.3	486	53	120	322	" "
16	15	30.2	3.25	9.3	33.5	15.5	14.3	4.5	465	57	106	283	" "
17	15	30.1	4.40	6.9	25.2	12.5	12.2	1.6	470	52	120	308	" "
27	15	34.4	5.30	6.5	23.0	12.5	8.0	1.5	475	57	95	303	
36	15	34.4	5.48	6.3	23.3	13.5	8.3	1.5	402	51	95	240	
57	15	38.4	6.84	5.6	18.0	8.6	7.3		345	41	65	234	
	Rabbit B. Weight 8½ lbs.												
1	30	32.4	5.17	6.3	21.7				375	28	83	287	Plasma clear.
2	30	23.3	3.57	6.5	20.3	9.7	8.0	1.3	365	29	80	281	" "
3	30	20.7	3.36	6.2	18.5	9.5	8.0	1.5	370	23	80	257	" "
4	30	18.4	3.19	5.8	23.0	12.0	10.9	±	430	14	109	316	Faint milkiness.
5	30	17.8	2.46	8.0	33.0	13.6	20.0		402	19	90	325	Plasma moderately milky.

TABLE I—*Continued.*

Day.	Blood taken.	Hematocrit.	Red count.	Corpuscle volume.	Phosphates per 100 cc.								Remarks.
					Plasma.				Corpuscles.				
					Total.	Inorganic.	Lipoid.	Organic.	Total.	Inorganic.	Lipoid.	Organic.	
Rabbit B—*Concluded.*													
	cc.	*per cent*	*millions*	*c.mm.* $\times 10^{-8}$	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
6	30	19.6	2.46	7.2	35.0	11.1	28.6	2.3	450	38	135	277	Plasma moderately milky.
7	35	17.3	1.71	10.0	40.0	10.0	29.0	±	422	21	121	284	Plasma milky.
8	30	17.5	2.32	7.5	50.0	11.1	37.5		470	32	145	288	" "
9	30	18.0	2.41	7.4	48.0	11.5	33.0	+	480	42	170	288	" "
10	15	16.1	2.20	7.4	57.0	6.0	43.0	4.8	475	43	155	317	" "
11	15	17.6	1.84	9.5	34.0	10.0	23.0	2.9	440	30	140	290	" "
12	15	20.2	2.37	8.5	45.0	13.0	32.0	±	430	57	150	233	" "
14	15	25.0	2.5	10.0	30.0	11.5	18.0	1.0	475	36	160	314	Faint milkiness.
15	15	24.1	2.52	9.6	30.0	12.0	19.0	0.8	430	20	170	260	" "
17	15	25.7	4.07	6.3	26.2	13.5	12.5		485	27	200	263	Very faint milkiness.
19	15	25.8	3.91	6.5	20.3	9.4	8.0	2.0	445	50	170	240	Plasma clear.
37	15	36.5	5.56	6.6	17.5	9.0	7.0	2.0	345	13	75	242	" "
Rabbit C. Weight 7½ lbs.													
1	20	36.0	5.40	6.6	14.0	7.5	7.5	1.3	315	8.5	63	262	Plasma clear.
4	25	22.5	3.96	10.6			10.0						Faint lipemia.
5	25	18.4	2.16	8.5	16.9	9.3	7.0	1.0	350	35.0	66	235	Plasma clear.
7	30	18.1	2.8	6.5	22.5	7.6	13.9	3.4	405	7.8	80	332	" faintly milky.
9	30	19.5	2.3	8.5	30.0	7.0	20.0	2.2	460	7.1	107	308	Plasma milky.
12	20	28.8	3.9	7.4	20.0	12.5	10.0	1.1	400	20.0	125	277	" faintly milky.
19	18	34.5	6.0	5.8	24.2	10.0	7.0	4.5	380	12.0	100	288	Plasma clear.
Rabbit E. Weight 8½ lbs.													
1	35	27.5	3.8	7.2	23.0	9.5	13.0	0.5	347	25.0	57	272	Plasma muddy but no lipemia.
3	35	25.3	2.74	7.2	21.0	6.6	12.5	3.1	325	9.3	55	261	" "
5	35	16.7	2.20	7.6	20.4	7.0	13.6	0.2	350	15.4	66	275	Plasma faintly milky.

TABLE I—*Concluded.*

Day.	Blood taken.	Hematocrit.	Red count.	Corpuscle volume.	Phosphates per 100 cc.								Remarks.
					Plasma.				Corpuscles.				
					Total.	Inorganic.	Lipoid.	Organic.	Total.	Inorganic.	Lipoid.	Organic.	
Rabbit E—*Concluded.*													
	cc.	*per cent*	*millions*	*c.mm.* $\times 10^{-}$	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
7	35	15.3	2.36	6.5	25.2	8.7	15.7	2.4	430	16.0	97	304	Plasma milky.
10	18	22.2	3.09	7.2	23.5	11.1	10.0	4.9	550	17.2	140	353	" clear.
16	18	29.9	4.77	6.3	22.5	14.3	8.5	2.2	465	16.5	97	333	" "
Rabbit F. Weight 5 lbs.													
1	20	36.0			33.1	17.5	14.3	2.0	415	65	63	285	Plasma muddy but no lipemia.
3	18	24.5	3.76	6.6	30.0	13.6	15.5	0.4	415	56	76	276	" "
5	20	21.3	2.87	7.6	40.0	13.7	21.5	4.3	470	56	104	266	Plasma faintly milky.
7	20	20.4		7.8	36.0	12.0	19.0	4.0	485	66	120	284	" "
15	14	30.8	3.97	7.8	30.0	15.0	11.1	2.3	470	47	100	343	Plasma clear.
Rabbit G. Weight 8 lbs.													
1	25	30.0	5.5	5.4	31.7		15.7		301		54		Plasma clear.
6	25	23.0	3.06	7.5	35.1		15.9		355				" "
7	20	26.4	2.80	9.4			25.4		364		94		" milky.
8	30	17.7	2.52	7.0			18.0		493		103		" "
11	25	28.3	3.76	7.5	33.8		12.8		374		100		" clear.

DISCUSSION.

Lipoid Phosphorus.

The most characteristic change is in the lipoid phosphorus. In the plasma it is always very much increased (up to five times its normal value), while in the corpuscles the increase is less marked, in only three cases rising to over twice the normal value. In the plasma the highest values for lipoid phosphorus are generally found at the height of the lipemia and diminish as the

visible milkiness of the plasma diminishes. But even when the plasma has become clear, high values for lipoid phosphorus persist for some days. In the corpuscles the highest values for lipoid phosphorus are not generally reached until some time after the height of the visible milkiness, or even after the plasma has been clear for a time and the values do not become normal until some days after the plasma values have become normal.

The plasma begins to show milkiness at about the time the blood corpuscles are reduced to half their original number. This generally persists throughout the period of low corpuscle values, although in one case the plasma cleared after the first milkiness although the number of corpuscles was lower than when milkiness first appeared.

It was found in earlier work (2) that lipoid phosphorus increased in the blood corpuscles during absorption of fat in dogs, and it seemed likely that the corpuscles took an active part in fat metabolism by the transformation of fat into lecithin.[1] In human anemia (Bloor and MacPherson (5)) it was found that abnormalities in the blood lipoids were present only when the blood corpuscles were reduced to below half their normal number, the abnormalities being ascribed to inadequate functioning of the red blood cells, due to their reduced number. In the results given above, increase of fat and its metabolites is found associated with low corpuscle values. Whether the same explanation holds—that the disturbance in fat metabolism, which shows itself in the lipemia, is directly the result of the inability of the smaller number of corpuscles to perform this function in fat metabolism—or whether the lipemia is indirectly connected with the corpuscles in some other way, must be left for future work to decide. The results obtained in the work on the above type of lipemia indicate again, however, the probability of a participation of the corpuscles in the metabolism of fat.

[1] Further unreported work in this laboratory has not, however, entirely borne out these findings, since certain dogs have been found, in which the increase of lipoid phosphorus took place in the plasma and not in the corpuscles.

Inorganic Phosphorus.

Plasma.—In Experiment A, with strong lipemia, inorganic phosphate increased to twice the normal value and remained high throughout. In Experiment B, also with marked lipemia, the inorganic increased, but not to more than 50 per cent above normal values. In Experiment C, with mild lipemia, increases of 70 per cent were found while in Experiment E, phosphate first diminished and then increased above the original value. In Experiment F, with mild lipemia, the inorganic phosphate diminished. In this case, however, it was unusually high to start with.

It thus appears that in severe lipemia increases of inorganic phosphates are to be found, while in the mild lipemia a slight increase or a decrease may be present. In none of the experiments was a parallelism between inorganic and lipoid phosphate to be noted. In a study of fat formation during milk production, Meigs and coworkers (4) found that with the decrease of lipoid phosphorus in the blood during its passage through the mammary gland there was a corresponding increase in inorganic phosphate resulting from the setting free of phosphoric acid from the lecithin in its change into fat. In cases where there was much increase of lecithin, as in the above, one might expect a decrease in inorganic phosphate in the plasma. Instead, there is found an increase. Inorganic phosphate in plasma must be subject, however, to constant change; loss through the excretions, and addition from food or from phosphate stores, and the same cause which produced an increase of lipoid phosphate might also act to increase the inorganic phosphate, either by retention or by supply from the stores, in order to provide the material for formation of lecithin from fat.

Another reason for expecting low rather than high values for inorganic phosphorus in the plasma is the formation of new blood cells, which are relatively very rich in phosphoric acid compounds. Diminution of inorganic phosphorus has actually been found in cases of a single withdrawal of blood (10). The same reasoning might be applied in this case as in the above—that with increased demand there is increased supply, and, as is usual in living beings, the supply is in excess of what was origi-

nally present. The only case in which the plasma values remained below the beginning value was in Rabbit F, where the beginning level was unusually high.

Corpuscles.—In the experiment with Rabbit B, the acid ammonium sulfate did not completely precipitate the protein of the corpuscles, hence the values of inorganic phosphate are doubtful. In all other cases protein was completely removed so that the figures given are probably correct. In Experiments A and B, with high lipemia, the inorganic phosphate increases more or less parallel with the lipoid phosphorus. In Rabbit C, with moderate lipemia, inorganic increased but irregularly. In Rabbit E, it diminished; in Rabbit F, it diminished slightly. It thus appears that unless the lipemia is marked the changes in values for inorganic phosphate in the corpuscles are slight or negative, while with marked lipemia the inorganic phosphate increases more or less parallel with the increase in lipoid phosphorus, and both are closely bound up with the appearance of the lipemia. An examination of Table I will show that in most cases high values for inorganic phosphorus in the corpuscles are accompanied by high values in the plasma, but there are many exceptions and nothing like a parallelism can be observed.

These results bring into discussion the whole question of the presence of free phosphates in blood. Taylor and Miller (11) found only traces which confirmed the earlier observation of Gürber (12). No water-soluble phosphate is extracted by the treatment of blood with alcohol-ether in the determination of lipoid phosphorus above, although phosphoric acid and water-soluble phosphates are measurably soluble in that solvent. In the results given above, there is rarely to be observed a balance or parallelism between inorganic phosphate in plasma and corpuscles. In rabbit as in human blood, the inorganic phosphate in the corpuscles is generally much higher than in the plasma. These observations render improbable any free exchange by diffusion of phosphate between corpuscles and plasma, such as would be expected if phosphate were present in the free form. On the other hand, Greenwald (13) and later Feigl (14), by treatment of the blood to remove protein with hot trichloroacetic acid, and the writer, by faintly acid, saturated ammonium sulfate, were able to get protein-free extracts which undoubtedly

contained free phosphate. Whatever objection may be raised to the use of hot trichloroacetic acid as being likely to break up unstable organic compounds of phosphoric acid cannot be urged as regards the cold treatment with the mildly acid ammonium sulfate and the inference is that inorganic phosphate must be present in the blood although not in a water-soluble, diffusible form. As was pointed out previously, there is enough calcium and magnesium in blood to combine with all the phosphate present, and at the prevailing reaction of the blood to form insoluble compounds with it. Another explanation which is in line with modern ideas is that the phosphate is combined with blood protein in such a way as to hinder or prevent its free diffusion. When the blood is deproteinized with reagents which readily dissolve phosphate—as with either of the acid reagents mentioned above—it is loosed from its protein combination and passes into solution. The fact that acid reagents dissolve phosphate while neutral reagents, as alcohol-ether, do not renders it probable that the phosphoric acid is present in a form insoluble in neutral solvent—as would be the case if combined with calcium or magnesium or with protein at the prevailing reaction of the blood.

Attention may be called to the fact that the amount of phosphate passing through the blood plasma daily is much greater than the amount constantly present. Thus the normal human being will excrete and normally consume with the food about 3 gm. of H_3PO_4 per day, while the amount present in the whole blood plasma would not be more than about one-eighth that amount.

Organic Phosphorus.

Corpuscles.—Organic phosphorus in corpuscles is subject in general only to small and irregular variations, indicating that this constituent is constant in the corpuscle from the time of formation, even under the stress of severe hemorrhage. The amount is also remarkably constant for all the animals reported here. The same may be said of the values obtained from a study of rabbit blood for another purpose, which included the examination of about twenty normal rabbits (10). Organic phosphorus in this amount appears to be characteristic of rabbit corpus-

cles. In the series reported above, notably in the case of Rabbit E and, to a certain extent, in Rabbits C and F, values of 15 or 20 per cent above the beginning values are found, but these serve only to emphasize the stability of values for this constituent. Low values are practically never found, and the high values may come at any time in the experiment after the anemia is established.

Plasma.—Organic phosphorus in the plasma is subject to great and irregular variations, so that very little can be said about it. In general, values are reached in the course of the bleeding which are much higher than those found at the beginning of the experiment. On the other hand, very low values are also found. No relation can be discovered between this constituent and the lipoid fraction, on the one hand, or the inorganic fractions, on the other. Normally, organic phosphorus has been found in the plasma of all the animals examined. During bleeding, it generally increases, but may disappear altogether at times, as in Rabbit B. It is possible that this constituent is connected on the one hand with the loss of the nucleus in the red cells, and with the presence of organic phosphorus in urine on the other.

Nucleoprotein, Etc.—In the normal animal, the sum of acid-soluble (inorganic and organic) and lipoid phosphorus is about equal (within the limits of error of the determinations) to the value of the separately determined total phosphate, so that the presence of notable amounts of other combinations of phosphorus—as, for example, nucleoprotein—is doubtful. In the course of the experiments it will be noted, however, that the sum of these fractions in both corpuscles and plasma is generally below the value for total phosphates, and sometimes markedly so, and it is therefore probable that some nucleoprotein is present during the anemia.

On the other hand, the sum of acid-soluble and lipoid sometimes gives a value notably higher than the total phosphate, which would indicate at those times some overlapping; *i.e.*, some unusual substance is present which dissolves in both the acid ammonium sulfate and the alcohol-ether.

Corpuscle Volume during Hemorrhage.—It is well known that in anemia corpuscles irregular both in shape and size appear and the abnormality in size is well illustrated in Column 5 of

Table I. The value "Corpuscle volume" was obtained by dividing the percentage volume of the corpuscles as shown by the hematocrit by the number, as obtained in the count. For example, in 1 c.mm. of blood, the number of corpuscles is known from the count while the volume which they occupy would be the fraction of 1 c.mm. represented by the percentage. Thus, with a count of 5.0 millions, and a corpuscle percentage of 31.0, the average volume of the individual corpuscle would be 0.31/5,000,000, or 6.2×10^{-8} c.mm. As may be seen from the table, the average corpuscle volume invariably rises during the course of the bleeding, sometimes to over 50 per cent above the beginning volume. As recovery takes place, the volume falls to the normal level for the animal or below it. Whether the increase in size is due to swelling of normal corpuscles, or whether some of the corpuscles are abnormally large when formed cannot be definitely determined from the data at hand, but the relative constancy of the value for organic phosphorus would indicate the latter.

Is there anything in the composition of the corpuscles to indicate whether they are young or old? Masing (15) from the results of similar experiments on rabbits and geese is of the opinion that the lipoid phosphorus content of the erythrocytes may be taken as a criterion of the age of the cells. In the work above the most marked difference between the composition of the corpuscles at the beginning and during the experiment, which might be taken as a criterion of the presence of newly formed corpuscles, is in the lipoid phosphorus content, which always rises during the hemorrhage and is the slowest of all constituents to return to normal values. The next most notable constituent is the organic phosphorus fraction, which also generally rises during the anemia. The increase above beginning values of this constituent is, however, relatively small and irregular. Whether these changes are due to the lipemia or are referable to the newly formed corpuscles cannot be stated. Present evidence on the dog indicates that in this animal, which apparently does not develop lipemia from bleeding, neither of these constituents is affected, which would lead one to the conclusion that changes in these values in the rabbit are referable to the lipemia.

While no exact relation has been found between the lipoid phosphorus and the other phosphorus compounds of the blood, it may be seen that in severe lipemia there is a marked increase of both inorganic and lipoid phosphorus in both plasma and corpuscles. Slight changes in lipoid phosphorus are not accompanied by corresponding changes in inorganic phosphorus.

SUMMARY AND CONCLUSIONS.

Of the phosphoric acid compounds of the blood, the one most markedly affected by anemia and consequent lipemia, is the lipoid phosphorus. Values up to five times the normal have been found in plasma, and to twice or over the normal value in the corpuscles.

When the lipoid phosphorus is markedly above normal in either plasma or corpuscles, there is an accompanying increase of inorganic phosphate, but no corresponding change in the other forms of phosphorus. Inorganic phosphorus thus appears to be the form most directly related to the lipoid phosphorus.

Organic phosphorus in the corpuscles remains remarkably constant throughout the experiments, the changes noted being relatively small, so that this constituent in the amounts noted appears to be characteristic of rabbit corpuscles from the time of formation. Organic phosphorus in the plasma varies greatly, sometimes being entirely absent. It generally increases during the bleeding.

The only form of phosphorus which is notably higher in newly formed corpuscles than in older ones is the lipoid phosphorus. Whether the higher values are characteristic of young corpuscles, or are due to the lipemia, cannot be stated.

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HEMATO-RESPIRATORY FUNCTIONS.

VII. THE REVERSIBLE ALTERATIONS OF THE H_2CO_3: $NaHCO_3$ EQUILIBRIUM IN BLOOD AND PLASMA UNDER VARIATIONS IN CO_2 TENSION AND THEIR MECHANISM.*

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It has been universally assumed that alterations of the gaseous and acid-alkali equilibrium of blood under the influence of variations in the tensions of oxygen and CO_2 are readily and completely reversible. The simplest conception of the process involved is as follows:

Hydrogen and chlorine ions (HCl) pass back and forth between the plasma and the corpuscles in response to variations of CO_2 tension, each time that the blood makes the round of the circulation. Thus Na in the plasma is released from NaCl to form $NaHCO_3$ as the blood comes to the tissues; and this Na is again neutralized to NaCl as CO_2 passes off in the lungs. The higher the tension of CO_2, and the concentration of H_2CO_3 in solution, the more Cl is displaced; and, in the proportion expressed by the CO_2 dissociation curve, the more $NaHCO_3$ is formed.

It follows from this that the tension of CO_2—at the moment, for instance, that the corpuscles are centrifuged off—determines (along a plasma curve dependent upon that of the whole blood) the quantity of alkali bicarbonate in the plasma. The power of separated plasma to vary its content of bicarbonate through the mediation of its proteins, although greater than that of a mere solution of $NaHCO_3$, is much less than that of whole blood.

* This and the following papers are continuations of work published by us in this *Journal* some months ago under the same general title (1). All these investigations were performed at about the same time (1918) and under the same conditions and collaboration.

This in brief is the present state of knowledge (2–5).

Some investigators (6, 7) have recently inclined to the view that hemoglobin itself is the principal CO_2-transporting agent. The evidence indicates, however, that hemoglobin, at least when in unlaked corpuscles, plays its part chiefly by absorbing acids and thus unmasking alkali from neutral salt to form bicarbonate. It is this action of the corpuscles which enables the plasma to transport CO_2. Probably also the same reaction occurs in respect to the fluid within the corpuscles since, under variations of CO_2 tension within physiological limits, and apart from the influence of oxygen, the corpuscles in normal blood take up and give off again (*in vitro*) an amount of CO_2 of about the same order of magnitude as an equal volume of the plasma.

We here intentionally avoid the question as to whether hemoglobin acts in the blood as an acid or as a base. We have therefore made use of a phraseology, *e.g.* "acid-concealing power, HCl load, and alkali-producing capacity of the corpuscles," compatible with any intracorpuscular mechanism—whether it finally turns out that hemoglobin acts by adsorption, or by chemical combination, as an acid or as a base or both.

If at the C_H prevailing in normal blood hemoglobin acts as an acid, as present theory seems to indicate (8), the absorption and concealment of HCl by the corpuscles may consist in some such reaction as: $Hb.B + HCl = Hb.H + BCl$. If on the contrary it acts as a base then presumably $Hb.OH + HCl = HbCl + H_2O$ or else $Hb + HCl = Hb.HCl$. It is certain, however, that it is not alkali hydroxide, but acid (chiefly HCl) which passes in and out between corpuscle and plasma.

Present information and variety of opinion on this and related topics may be seen in the papers of Davies, Haldane, and Kennaway (9), Gray (10), Joffe and Poulton (11), Campbell and Poulton (12), Dale and Evans (13), and Mellanby and Thomas (14). All these papers reached us after our own were ready for press. Many of the observations of these writers agree, but one paper (14) disagrees decidedly in its interpretation, with ours.

The peculiar feature of our discussion is that we have thought of the blood as a system which, when it has no tension of CO_2, contains no alkali bicarbonate. Starting from this assumption (verifiable by the experiment of evacuating the blood in a vacuum) we are justified, we think, in treating any $NaHCO_3$ which occurs in blood as called forth by the existing tension of CO_2 by action on the corpuscles.

Thus hemoglobin is almost as essential for the transportation of CO_2 by blood as it is for the transportation of oxygen, but its modes of action in relation to the two gases are quite different.

The following experiments illustrate the reversible reaction under the influence of CO_2. They will serve us as a background for dealing in succeeding papers with an irreversible alteration of the CO_2 ratio, and its bearing upon the interaction of oxygen and CO_2 in blood and upon hemolysis. Our experiments on these topics are published now, not because we can as yet fully explain the phenomena, but because of the interest which this general field of work is exciting, and because investigators unaware of this peculiar irreversible effect may be led to quite erroneous conclusions regarding some phases of the hemato-respiratory functions.

The normal reversibility of the CO_2-alkali equilibrium may be illustrated as follows:

Experiment 1.—Blood was drawn through a cannula from the femoral artery of a dog under local anesthesia with cocaine, and was delivered under mineral oil in a test-tube, where it was mixed with 0.005 gm. of ammonium oxalate per cc.; *i.e.*, 0.5 per cent.

It was divided into two samples which were treated as follows:

Sample A was equilibrated at 37°C. with air containing 70 mm. of CO_2. A part was then analyzed for its CO_2 content. The remainder of this sample was then equilibrated with 40 mm. of CO_2 and analyzed.

Sample B was equilibrated in succession with the same tensions of CO_2, but in reverse order.

The analyses showed CO_2 contents as follows:

Sample A.	At 72 mm.	=	56	volumes	per cent	of	CO_2
	" 40 "	=	46	"	"	"	" CO_2
Sample B.	" 40 "	=	46	"	"	"	" CO_2
	" 72 "	=	56	"	"	"	" CO_2

(This demonstration of perfect reversibility may seem, now at least, so perfectly obvious as not to deserve publication, but it is essential as a control for comparison with the observations to be reported in succeeding papers.)

The Behavior of Plasma in Contact with Corpuscles and After Separation.

As long as a plasma forms part of a normal blood and is in contact with its corpuscles its content of bicarbonate rises and falls, within physiological limits of CO_2 tension and apart from the oxygen effect, in close parallel with the CO_2 content; that is, with the dissociation curve of the whole blood. Above normal limits of CO_2 tension the plasma takes up less than its share of

any increase of CO_2 content, and further absorptions of CO_2, aside from mere solution, are effected almost wholly by the corpuscles.

From any blood there may be obtained many plasmas of widely varied bicarbonate content, and each of these plasmas has, after separation from the corpuscles, its own particular form of behavior; that is, its own dissociation curve. The plasma proteins are evidently able to function like the corpuscles in providing alkali, but less effectively. Thus there is a primary plasma curve, which within certain limits of CO_2 tension closely parallels that of the whole blood when normal, but not when abnormal. This curve is obtained by centrifuging the blood at various tensions of CO_2 and analyzing the plasmas at these tensions. But each of these plasmas, when exposed to various tensions of CO_2 after separation from corpuscles has a curve of its own, and (as has been shown previously by Hasselbalch and Warburg (15)) these secondary curves are flatter than the primary curve and cross it at the CO_2 tensions at which the blood was centrifuged.

Experiment 2.—Oxalated dog blood was divided into two samples which were equilibrated at 37°C. to tensions of CO_2 of 40 and 58 mm. respectively. The CO_2 content of the blood was then in each case determined. The plasmas were separated by the centrifuge, at 37°C. and under the equilibrating atmospheres, and their bicarbonate contents were determined.

	CO_2 tension.		Rise in CO_2 content.
	40 mm.	58 mm.	
	vol. per cent	*vol. per cent*	*vol. per cent*
Bicarbonate content of 1 cc. of whole blood	46	52	6
" " " 1 " " plasma.....	58	64	6
Difference between blood and plasma.....	12	12	0

It is here seen that, within the limits of the experimental conditions, the CO_2 content of the plasma has a constant difference from the CO_2 content of the whole blood from which it is separated, and that the bicarbonate content of the plasma is controlled by the CO_2 tension in the blood at the moment of separation. In blood drawn from various individuals (dogs) we find that the plasma-blood difference has various values, the figures for the

plasma being sometimes lower and sometimes higher than for the whole blood. But in all normal bloods the type of relation here shown holds true; that is, within physiological limits of CO_2 tension the plasma-blood difference is nearly constant. This seems to indicate that, apart from the change in volume of the corpuscles, and apart from the oxygen effect, the plasma and the corpuscles, at all moderate tensions of CO_2, take an equal share in the absorption and release of CO_2. This accords fully with the observations of Van Slyke and Cullen (4) on this important point.

This relation of plasma and corpuscles holds true so generally that we have used it as a test that the sample under study is normal resting blood. Blood is easily altered in this respect; for example, by overaeration in the process of defibrination, by gaseous evacuation, and similar treatments involving the irreversible effect described in succeeding papers.

In the next two experiments it will be seen that the plasma not only varies in its CO_2 content according to the tension of CO_2 at which it is separated from its corpuscles, but also that when these plasmas—all from the same blood—are equilibrated with various tensions of CO_2 each has its own dissociation curve.

Experiment 3.—Oxalated dog blood was divided into three portions which were then equilibrated with air containing CO_2 at 21, 40, and 73 mm., respectively at 40°C. 1 cc. of each was analyzed and the remainder of each was centrifuged. The plasmas from the first and third (the second was lost) were then equilibrated with CO_2 at 21, 40, and 73 mm. at 40°C. and analyzed. The results obtained (after subtraction of dissolved CO_2) were as follows:

	CO_2 tension.		
	21 mm.	40 mm.	73 mm.
	vol. per cent	*vol. per cent*	*vol. per cent*
Whole blood	38	49	57
Plasma separated at 21 mm.	40	47	52
" " " 73 "	45	53	58

Experiment 4.—Oxalated dog blood was divided into three samples and treated as in the previous experiment. The (=) sign is used to signify "contained." The amount of combined CO_2 is stated after it.

Sample A. At 21 mm. CO_2 = 32 volumes per cent CO_2, its plasma = 35 volumes per cent CO_2, and at 21 mm. CO_2 = 35 volumes per cent CO_2
40 " CO_2 = 39 " " " CO_2
72 " CO_2 = 45 " " " CO_2

Sample B. At 40 mm. CO_2 = 40 volumes per cent CO_2, its plasma = 44 volumes per cent CO_2, and at 21 mm. CO_2 = 41 volumes per cent CO_2
40 " CO_2 = 44 " " " CO_2
72 " CO_2 = 51 " " " CO_2

Sample C. At 72 mm. CO_2 = 51 volumes per cent CO_2, its plasma = 54 volumes per cent CO_2, and at 21 mm. CO_2 = 47 volumes per cent CO_2
40 " CO_2 = 51 " " " CO_2
72 " CO_2 = 54 " " " CO_2

These data are expressed graphically in Fig. 1.

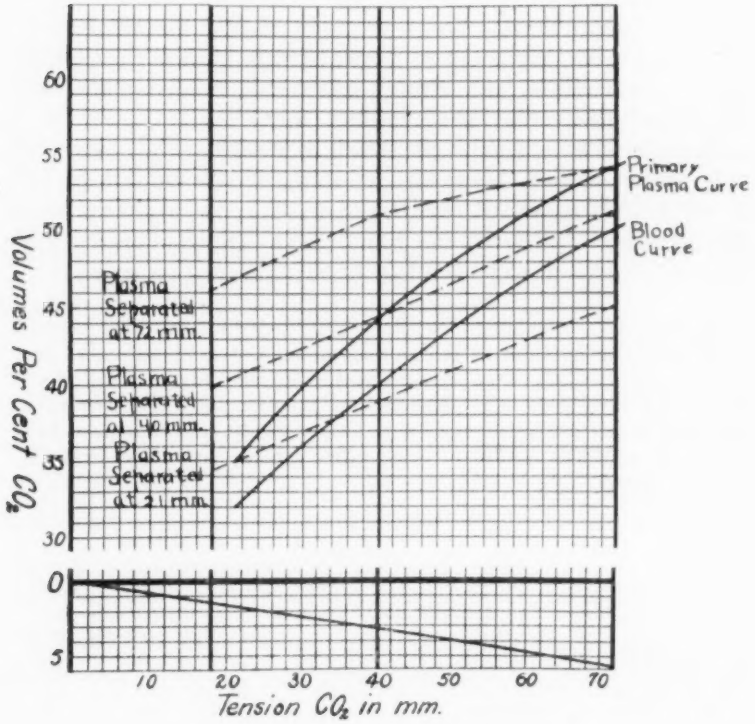

FIG. 1. From the data of Experiment 4.

The limit of the plasma-corpuscle interplay of chlorine is shown in the next experiment. Here the passage of chlorine into the

corpuscles and the unmasking of sodium in the plasma to form $NaHCO_3$ is seen to become inadequate between 74 and 140 mm. of CO_2. The curve for the whole blood and that for the plasma then become divergent, and the difference between the CO_2 contents of blood and plasma increases. The plasma at such tensions would do much less than its share in the transportation of CO_2. Up to 74 mm., the relation formulated in connection with Experiment 2 still holds true, however.

Experiment 5.—Oxalated dog blood was divided into four portions, which were equilibrated separately with tensions of CO_2 of 42, 74, 140, and 360 mm. CO_2 respectively. Part of each was analyzed; and the remainder was centrifuged and its plasma analyzed. The results were as follows:

	CO_2 tension.			
	42 mm.	74 mm.	140 mm.	360 mm.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*
Combined CO_2 of whole blood	47	53	75	110
" CO_2 " plasma	49	55	70	93
Difference between blood and plasma	+2	+2	−5	−17

Experiment 6.—Oxalated dog blood was divided into seven samples and equilibrated at 40°C. at the tensions of CO_2 shown in the table. A part of each sample was analyzed directly and the remainder centrifugalized under oil and its plasma analyzed.

	CO_2 tension at 40°C.								
	21 mm.	41 mm.	72 mm.	146 mm.	210 mm.	360 mm.	440 mm.	504 mm.	712 mm.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
Whole blood	30	42	54	66	72	77	80	79	80
Plasma	34	46	56	61	61	60	62	60	58

From Experiment 6 it appears that when blood is exposed to increasing tensions of CO_2 the amount of alkali produced in the plasma reaches a maximum between 72 and 146 mm. and that the amount of CO_2 combined by the corpuscles reaches its maximum somewhat below 440 mm.

Parallelism of Primary Plasma Curve and Whole Blood.

The parallelism of the primary plasma curve and that of the whole blood means simply that, under the influence of changes of CO_2 tension (but with uniform oxygen), the concentration of alkali bicarbonate rises and falls equally inside the corpuscles and in the plasma surrounding them. If this were not the case, the C_H inside the corpuscles and that in the plasma would be influenced unequally by variations of CO_2 tension. (We are here neglecting the influence of oxygen upon CO_2 capacity and we are assuming that the only condition determining C_H is the CO_2 ratio, H_2CO_3:$NaHCO_3$.)

In altered blood in which this parallelism does not hold, either such differences of C_H inside and outside the corpuscles are developed or the hemoglobin undergoes some alteration of its acid-absorbing power, such as an increased capacity for interaction of oxygen and CO_2.

Relation of Plasma Alkali to HCl Load of Corpuscles.

It appears also from the foregoing experiments that it is quite misleading to think of the plasma as having, independently of the corpuscles, a certain alkali value. Just as an organism dominates its environment, so the corpuscles control the concentration of bicarbonate in the fluid around them. They vary it under the influence of variations in CO_2 tension and C_H. When the plasma alkali is determined by analysis, what is revealed is fundamentally the alkali-producing power of the corpuscles and the load of HCl which they are capable of taking. As will be shown in the next paper, corpuscles which have the quality of inducing, at a certain tension of CO_2, a certain amount of alkali—be it large or small—induce nearly that amount of "alkaline reserve" even in a normal saline solution.

The enormous buffer value of hemoglobin endows the blood with a potential alkaline reserve which is much larger than the plasma alkali. The plasma alkali represents merely that fraction of the alkali-producing power which is called into use at a certain tension of CO_2, concentration of H_2CO_3, and C_H.

CONCLUSIONS.

Hemoglobin plays almost as large a part in the transportation of CO_2 as it does in that of oxygen. It does not itself combine directly with CO_2 to any great extent, however; but under pressure of CO_2 it absorbs HCl, and thus provides alkali, which in the plasma is chiefly Na from NaCl, to form bicarbonate. As CO_2 is given off in the lungs, NaCl is reformed from $NaHCO_3$.

From any blood, therefore, plasmas of widely varying content of alkali are obtained, depending upon the tension of CO_2 at the time of centrifuging. Each of these plasmas has various alkali values with varied tensions of CO_2. The relations of the primary and secondary dissociation curves of the plasma to each other and to the curve of the whole blood are here exemplified.

A quality of normal blood from a resting animal is that variations of CO_2 tension (in the presence of ample oxygen) call nearly the same increase of alkali into use in unit volume of the blood as in unit volume of its unseparated plasma. Thus the bicarbonate content of the corpuscles (apart from the effect of variations of oxygen) rises and falls with that of the plasma around them. But this does not hold at abnormally high tensions; and at very low tensions, as will be shown in later papers, the blood undergoes an irreversible alteration. The parallelism of alkali variations in corpuscles and plasma affords, therefore, a convenient test as to whether or not a given blood sample is normal.

It is probably by means of the equality of gain and loss of bicarbonate that the C_H inside the corpuscles and that outside them in the plasma are kept the same, or at least in uniform relation. The C_H indicated by the primary plasma curve at various tensions of CO_2 in the blood is particularly significant.

It thus appears that the corpuscles dominate the plasma so powerfully that the alkali of the (unseparated) plasma at a certain tension of CO_2 is essentially an expression of the alkali-producing power of the corpuscles. The plasma alkali is only a part of the total alkaline reserve of the blood. The chief buffer is the hemoglobin. The extent to which the corpuscles are loaded with HCl is indicated by the amount of alkali which they call into use out of NaCl at a certain tension of CO_2; that is, the CO_2 capacity of the blood.

The condition underlying the variations in this quality of the corpuscles will be considered in the next paper.

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HEMATO-RESPIRATORY FUNCTIONS.

VIII. THE DEGREE OF SATURATION OF THE CORPUSCLES WITH HCl AS A CONDITION UNDERLYING THE AMOUNT OF ALKALI CALLED INTO USE IN THE PLASMA.

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It is shown in the preceding paper that the alkali of the plasma, chiefly $NaHCO_3$, is not an independent property of that fluid, but is controlled and determined by the corpuscles. From this it follows that when a figure for the plasma alkali, for instance 40, 55, or 70, is found by analysis, its significance for the blood lies in large part in what it tells of the quality of the corpuscles.

In this paper we shall report experiments which throw some light on this quality of the corpuscles. What are the conditions which determine why one blood, when equilibrated with a certain tension of CO_2 (for instance 40 mm.), develops a plasma alkali of 40, while another blood at the same tension develops 55, and a third 70? The experiments given below indicate that one of the conditions in question is the extent to which the corpuscles are loaded with HCl.

It will here appear that the amount of alkali developed in the plasma of any blood equilibrated to 40 mm. of CO_2 tension is an index of the extent to which the corpuscles have been previously depleted of alkali-producing power. If they are heavily loaded already they can take additional HCl less readily, and thus develop less alkali in the plasma than if previously only lightly loaded. If on the contrary the load of the corpuscles is artificially decreased, by the treatment described below, they will then take HCl more readily and thus produce a higher plasma alkali than they were previously able to do. Thus, as we shall show, any corpuscles may be made to furnish any plasma alkali.

The underlying reasons for this behavior of corpuscles are probably to be found (1) in the capacity of hemoglobin to combine with large amounts of acid, or alkali, under slight change of C_H—in other words its extraordinary buffer value; and (2) in the tendency of normal corpuscles to maintain within certain limits the same C_H, or at least the same difference of C_H, in the fluid around about them and in their own intrinsic fluid under variations of CO_2 tension.

To Zuntz (1) and to Hamburger (2) we owe the clear recognition and demonstration of the fact that under the influence of CO_2 there is a passage of HCl between plasma and corpuscles. Gürber (3), Van Slyke and Cullen (4), and Fridericia (5) have proved by chloride analyses that the amount of HCl formed from NaCl and passing into the corpuscles corresponds to the greater part of the amount of alkali bicarbonate produced.

Starting from this point, our experiments show the really enormous amounts of HCl which the corpuscles are capable of taking up under slight increase of the CO_2 ratio and C_H. These observations illustrate the effectiveness with which the corpuscles stabilize this ratio ($H_2CO_3 : NaHCO_3$), and therefore the C_H, in the plasma. The conception of the "HCl load of the corpuscles," here introduced, suggests that the real source of the alkali of the plasma is not the alkali of the food, but the NaCl of the blood. It follows from this conception that, for instance, if two plasmas, separated in both cases under 40 mm. of CO_2, show CO_2 contents respectively of 50 and 60 volumes per cent, the HCl loads of the corpuscles in the two cases must differ by an amount very much greater than a quantity of HCl which would correspond to 10 volumes per cent of CO_2. From this conception we may safely infer that when the body is striving to call alkali into use in the plasma through depressed breathing and acidosis, or to cause alkali to disappear through acapnia and alkalosis, the variations induced in the HCl load of the corpuscles must be very large indeed—much greater than analyses of plasma CO_2 would directly indicate.

The method of experimentation was as follows: Samples of oxalated dog blood were equilibrated with various tensions of CO_2 and the plasmas separated by the centrifuge. The corpuscles were then washed once with normal saline solution (0.9

per cent NaCl solution with no tension of CO_2), recentrifuged, and then made up to the original blood volume with fresh saline solution. The resulting suspension was thoroughly mixed, and was then equilibrated with CO_2 at various tensions. Finally the saline solution was separated from the corpuscles by means of the centrifuge; and the bicarbonate which had been formed in the saline was determined.

All the operations of equilibration, centrifuging, and analyzing were carried out at a summer room temperature of 30°C.

Experiment 1.—

	Combined CO_2 in Sample A.		Combined CO_2 in Sample B.
	vol. per cent		*vol. per cent*
(1) Blood equilibrated with 41 mm. CO_2 and centrifuged.		(1) Another sample of same blood equilibrated with 712 mm. CO_2 and centrifuged.	
Its plasma at 41 mm...	48	Its plasma at 41 mm...	80
(2) Corpuscles (from A1) washed, mixed with saline, and equilibrated with 41 mm. CO_2.		(2) Corpuscles (from B1) washed, mixed with saline, and equilibrated with 41 mm. CO_2.	
Corpuscle-saline suspension	32	Corpuscle-saline suspension.....	22
Saline centrifuged off...	46	Saline centrifuged off....	29
(3) The same corpuscle-saline suspension (as A2) equilibrated at 712 mm. CO_2 and centrifuged.		(3) The same corpuscle-saline suspension (as B2) equilibrated at 712 mm. CO_2 and centrifuged.	
This saline at 41 mm. CO_2.....	77	This saline at 41 mm. CO_2.....	40
Sum of (1) and (3)	125		120

In Experiment 1 it is to be noted that (in Samples A1 and A2) the figures for the alkali called into use by these corpuscles at 41 mm. of CO_2 in the natural plasma and in a saline solution are nearly the same; namely, 48 and 46 volumes per cent of CO_2.

Note that similarly (in Samples A3 and B1) equilibration at 712 mm. of CO_2 also calls into use nearly the same amount of alkali from natural plasma and from saline; namely, 77 and 80 volumes per cent of CO_2.

Note on the other hand that after the corpuscles have received an increased load of HCl under the influence of a high tension of CO_2 (as in Sample B1) their power of calling alkali into use is diminished, for example from 46 (in Sample A2) to 29 (in Sample B2) and from 77 (in Sample A3) to 40 volumes per cent (in Sample B3).

We have found in other experiments, in which the CO_2 dissociation curve of whole blood was determined up to very high tensions (700 mm. and over), that above certain limits no more alkali is called into use, and additional CO_2 is taken up only in simple solution (see Experiment 6 of the preceding paper). From this it might be supposed that the total alkali-producing power of the corpuscles had been called into use, and that the combined CO_2 at, for instance, 450 mm. could be taken as a measure of it.

But from Experiment 1 it appears that such is not the case; for the same corpuscles which (in Sample B1) produced an alkali of 80 in their own plasma at 712 mm. had still the power to produce considerable additional amounts of alkali in saline (Samples B2 and B3). Evidently the alkali-producing force is balanced against the alkali (or the C_H) already in the fluid, either plasma or saline. (Quantitatively there are discrepancies in our data from whole blood, in the preceding paper, and from corpuscle-saline suspensions.)

Experiment 2.—Blood (from same dog as in Experiment 1) equilibrated with 712 mm. of CO_2, and centrifuged as in Sample B of Experiment 1.

The corpuscles were then mixed with saline to which a small amount of sodium bicarbonate had been added. The suspension was then equilibrated with 41 mm. of CO_2 and centrifuged.

Saline-bicarbonate solution at 41 mm. CO_2 before addition of corpuscles = 49 volumes per cent CO_2.

Saline-bicarbonate solution after centrifuging = 10 volumes per cent CO_2.

Experiment 3.—

Sample A. Arterial blood centrifugalized.

Plasma bicarbonate at 41 mm. = 36 volumes per cent CO_2.

Sample B. The corpuscles (from Sample A) mixed with saline and equilibrated with 41 mm. CO_2.

Saline centrifuged off at 41 mm. = 33 volumes per cent CO_2.

Sample C. The corpuscles (from Sample B) were again mixed with fresh saline, and again equilibrated to 41 mm. CO_2, and centrifuged.

This saline at 41 mm. = 28 volumes per cent CO_2.

Sample D. The corpuscles (from Sample C) were mixed with saline a third time, equilibrated to 712 mm. CO_2, and centrifuged.

This saline at 41 mm. = 54 volumes per cent CO_2.

Total of plasma and salines = 151 volumes per cent CO_2.

(The equal sign (=) is here used to signify that the saline or plasma was found on analysis to contain the indicated amount of combined CO_2; *i.e.*, $NaHCO_3$.

Experiment 4.—

	Combined CO_2 in Sample A.		Combined CO_2 in Sample B.
	vol. per cent		*vol. per cent*
(1) Blood rotated for a few minutes in a flask with room air and centrifuged.		(1) Another sample of same blood equilibrated at 712 mm. CO_2 and centrifuged.	
Plasma at 41 mm. CO_2..	35	Plasma at 41 mm. CO_2.	91
(2) Corpuscles (from Sample A1) washed, mixed with saline, and equilibrated at 41 mm. CO_2.		(2) Corpuscles (from Sample B1) washed, mixed with saline, and equilibrated at 41 mm. CO_2.	
Corpuscle-saline suspension.....	35	Corpuscle-saline suspension.....	22
Saline centrifuged off...	50	Saline centrifuged off..	36
(3) Corpuscles (from Sample A2) suspended in fresh saline, equilibrated with 712 mm. CO_2 and centrifuged.		(3) The same corpuscle-saline suspension (as Sample B2) equilibrated with 712 mm. CO_2 and centrifuged.	
This saline at 41 mm. CO_2.....	104	This saline at 41 mm. CO_2.....	91
Total of plasma and salines.	189	Sum of (1) and (3).....	182

Experiment 5.—Arterial blood was used. It contained 40 volumes per cent of CO_2. It was centrifuged, and the corpuscles were found to constitute almost exactly 50 per cent of the volume. The plasma contained 40 volumes per cent of CO_2.

The corpuscles were divided into four parts and suspended respectively in saline solution (0.8 per cent NaCl), (*a*) of equal volume, (*b*) of three volumes, (*c*) of six volumes, (*d*) of twelve volumes. All were equilibrated at 40 mm. of CO_2 at 37°C. and centrifuged; and the salines were analyzed.

	CO_2 in 1 cc.	Total combined CO_2 produced by 1 cc. corpuscles.	CO_2 ratio.	$C_H 7$	pH
	vol. per cent	*vol. per cent*			
Plasma	40	40	3:40	0.60	7.22
Saline (*a*), one volume	32	32	3:32	0.75	7.12
" (*b*), three volumes	27	81	3:27	0.88	7.05
" (*c*), six "	24	144	3:24	1.00	7.00
" (*d*), twelve "	19	228	3:19	1.26	6.91

From these data we see that under an increase of C_H from 0.6 to 1.26 $\times 10^{-7}$ (or pH 7.22 to 6.91) these corpuscles absorbed an amount of HCl, and produced an amount of alkali, corresponding to 228 volumes per cent of CO_2.

From Experiment 2 we see that when corpuscles have been loaded with HCl by exposure to a high tension of CO_2 and are then transferred to a saline solution of moderate alkalinity at a moderate tension of CO_2, they give off HCl and reduce the alkali of the solution, turning $NaHCO_3$ into NaCl.

From Experiment 3 we see that normal corpuscles have a very large capacity for HCl. The figures show only a slight decrease of alkali production in the second saline in which they were equilibrated at normal CO_2 tension. That they still had a large reserve capacity for HCl is shown by the even larger amount of alkali produced in a third saline in which they were equilibrated with a high tension of CO_2.

In Experiment 4 the contrasting figures for Samples A and B show that corpuscles may have their load of HCl decreased by equilibration to a low tension of CO_2, and increased by a high tension. The less loaded and the more loaded are then seen to produce quite different amounts of alkali in saline both at normal and at supernormal tensions of CO_2. But the increase of alkali called forth is nearly the same. Thus comparing Sample A2 with Sample B2, and Sample A3 with Sample B3 we find $50 - 36 = 14$, and $104 - 91 = 13$.

From these experiments it appears that the capacity of the corpuscles to absorb and give off HCl is very large, and that it is controlled by the tension of CO_2 through the concentration of H_2CO_3 in solution. Evidently the total alkaline reserve of the blood is not merely the $NaHCO_3$ of the plasma but the entire acid-absorbing and alkali-producing power of the corpuscles. The data above given indicate that this acid-concealing power of the corpuscles is five or ten times as great as the acid-neutralizing power of the $NaHCO_3$ in its plasma.

Regulation of Plasma C_H by Alkali-Producing Power of Corpuscles.

We thus see the enormously important part played by the corpuscles in the regulation of C_H in the plasma. Indeed, the intrasanguinary mechanism for the regulation of neutrality need involve little more than the capacity of the corpuscles to produce $NaHCO_3$, or to neutralize it to NaCl, almost in proportion to the concentration of H_2CO_3; for theory indicates that the C_H varies in proportion to the CO_2 ratio ($H_2CO_3 : NaHCO_3$).

Consideration of these experiments suggests that the placing of corpuscles in saline is virtually equivalent to adding to blood an amount of HCl just sufficient to convert all the $NaHCO_3$ of the plasma into NaCl. Presumably, if the blood were then equilibrated with 40 mm. of CO_2 the corpuscles would in large part (four-fifths to nine-tenths) restore the alkali in the plasma. But the C_H would be increased by 10 or 20 per cent.

Doubtless it is through its influence upon C_H that the tension of CO_2 and concentration of H_2CO_3 act. If so, the effects of the addition of acid or alkali to blood should be dealt with, not at a uniform tension of CO_2, as in the experiments above reported, but at a uniform C_H; that is, at a uniform CO_2 ratio. Thus if the normal level of the ratio $H_2CO_3 : NaHCO_3$ is 3 : 60, and an amount of HCl is added corresponding to 20 volumes per cent of CO_2, the plasma alkali would not fall from 60 to 40 (although $60 - 20 = 40$), but merely to an intermediate figure, as long as the tension of CO_2 is unchanged. But if the tension were reduced so that the concentration of H_2CO_3 is now 2 instead of 3, the alkali should then be 40. At the new level of 2 : 40 the C_H would again be normal. The load of HCl on the hemoglobin—or, if hemo-

globin acts as an acid, the amount of base combined with it—would then be the same as at the original 3 : 60 level. The whole change would consist in the neutralization of part of the alkali in use and a proportional reduction of H_2CO_3. The CO_2 ratio and C_H would be unaltered. This is, apparently, what occurs *in vivo* (6).

The same consideration would apply to a rise of level to 4 : 80 due to addition of alkali. The CO_2 ratio and C_H being normal, the load on the hemoglobin would be the same as at the levels 2 : 40 and 3 : 60.

We have performed some experiments *in vitro* which suggest that these expectations are correct, but they need to be repeated and extended before detailed publication.

CONCLUSIONS.

Centrifuged corpuscles suspended in saline solution and exposed to a tension of CO_2 produce in the saline solution an amount of alkali only a little (one-fifth or one-tenth) less than that called into play in the plasma before centrifugation at this CO_2 tension.

Corpuscles may have their load of HCl increased or decreased by suitable equilibrations. The alkali-producing power of the corpuscles is adjusted accordingly. When suspended in a saline solution, corpuscles already heavily loaded with HCl call forth less alkali at a given tension of CO_2 than less loaded corpuscles.

The total capacity of the corpuscles for acid is equivalent to an amount of alkali several times greater than that in the plasma of normal blood. This capacity is controlled immediately by the tension of CO_2 and concentration of H_2CO_3 and presumably the C_H, in the blood. When the H_2CO_3 is increased there is an almost proportionate increase of $NaHCO_3$ and the change in the CO_2 ratio ($H_2CO_3 : NaHCO_3$) and C_H is correspondingly slight.

When whole blood is equilibrated at a very high tension of CO_2 the alkali-producing power is not (as might be supposed) exhausted. The alkali called into use at such tensions is therefore not a measure of the total potential alkali-producing capacity of the corpuscles. It is merely a measure, or approximation, of what they can do in the existing plasma (high in $NaHCO_3$), against which they are balanced.

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HEMATO-RESPIRATORY FUNCTIONS.

IX. AN IRREVERSIBLE ALTERATION OF THE H_2CO_3:$NaHCO_3$ EQUILIBRIUM OF BLOOD, INDUCED BY TEMPORARY EXPOSURE TO A LOW TENSION OF CO_2.

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Observations in this laboratory have pointed repeatedly to the occurrence of a more or less permanent and irreversible, or difficultly reversible, alteration in the blood under low tensions of CO_2. Henderson (1) observed such an alteration *in vivo* in 1908. Henderson and Haggard (2) estimated the critical level at 33 to 36 volumes per cent of CO_2 in the arterial blood, but we now think that, owing to neglect of temperature, these figures are somewhat too high.

We found that in dogs, when overventilation of the lungs was carried so far that the arterial blood was reduced below the critical level, an irreversible reduction in the CO_2-combining power of the blood occurred. Thereafter, restoration of CO_2 to the blood even by inhalation of CO_2 in air was ineffective, and death always followed.

In a more recent publication (3) we have called attention to the fact that in equilibrating blood with gases, for the determination of the CO_2 dissociation curve, care must be taken that the blood is not exposed to very low tensions of CO_2; *e.g.*, 20 mm. or less at body temperature. At such tensions we found that the same, or a closely similar, alteration of the blood occurs *in vitro* as that noted above *in vivo*.

We were led to this observation by the occurrence of discrepancies in our analytical results. In some experiments the CO_2 content deduced from the CO_2 dissociation curve of the blood at the alveolar CO_2 tension did not agree with the CO_2 content found

in the arterial blood by direct analysis. This indicated that in these cases the curve was erroneous, and that the error was due to alterations in the blood *in vitro*.

In Experiment 1 is to be seen an example of this type of analytical discrepancy. The arterial blood contained 46 volumes per cent of CO_2. Some of this same blood equilibrated with air containing 41 mm. of CO_2 likewise contained 46 volumes per cent of CO_2. But when a third sample of the same blood was equilibrated in succession to tensions of 18, 40, and 72 mm., the curve plotted from the analytical results indicated a CO_2 content of only 42 volumes per cent at 41 mm. Evidently the blood had lost CO_2 capacity in the course of the equilibrations; that is, it called less alkali into use at a CO_2 tension of 41 mm. than originally.

Experiment 1.—The femoral artery of a dog was cannulated under local anesthesia. Arterial blood was drawn under oil and onto a small amount of oxalate. It was divided into three samples of which one (*a*) was immediately analyzed, another (*b*) was equilibrated with air containing 41 mm. CO_2 at 37.5°C. and analyzed, and the third (*c*) was equilibrated in succession at 37.5°C. with air containing 18, 40, and 72 mm. CO_2, and an analysis made at each tension.

The analyses showed (*a*) = 46 volumes per cent CO_2; (*b*) = 46 volumes per cent CO_2; (*c*) at 18 mm. = 30 volumes per cent CO_2, at 40 mm. = 41 volumes per cent CO_2, and at 72 mm. = 50 volumes per cent CO_2.

From the dissociation curve plotted from the last three figures, (*c*) at 41 mm. would contain about 42 volumes per cent CO_2 in contrast to 46 in (*a*) and (*b*).

It needs to be distinctly noted that this indicates quite a different type of alteration of the corpuscles from the immediately reversible reaction to CO_2, or the adjustment of their load of HCl, discussed in the two previous papers.

Experiment 2.—Arterial blood from a dog was oxalated. It was then equilibrated at 18 mm. of CO_2, at 37°C. for ½ hour. At intervals samples were taken and analyzed for their content of CO_2. As 8 minutes is ample time for equilibrium, the following figures suggest a progressive loss of CO_2-combining power.

Time, *min*	0	8	15	20	30
CO_2 content, *vol. per cent*	45	26	24	22	21

Observations of the character shown in Experiment 2 also led us to suspect that exposure of blood *in vitro* to low tensions of CO_2 results in some fundamental alteration in its CO_2-combining

power. To test this hypothesis a sample of blood was equilibrated in succession with decreasing tensions of CO_2 and its dissociation curve plotted. With a second portion of the same blood equilibrations were made in the reverse order; that is, beginning with a very low tension but using the same gas mixtures as previously. The results obtained are shown in Experiment 3.

Experiment 3.—Oxalated arterial dog blood was used. One sample (*a*) was equilibrated at 37.5°C., for 15 minutes each time, with air containing 72, 40, 18, and 1 mm. CO_2, and an analysis for CO_2 content was made at each tension. A second sample (*b*) was similarly equilibrated with the same CO_2 tensions but in reverse order. The analysis showed:

Sample (*a*) at 72, 40, 18, and 1 mm. contained 56, 47, 32, and 14 volumes per cent CO_2 respectively.

Sample (*b*) at 1, 18, 40, and 72 mm. contained 14, 27, 38, and 49 volumes per cent CO_2 respectively.

(Previous experiments have demonstrated that the time, 15 minutes, here allowed, is quite sufficient for the complete attainment of equilibrium.)

It has been shown by Christiansen, Douglas, and Haldane (4) that the CO_2-combining power of defibrinated blood is sometimes initially greater than that of the undefibrinated, and that the defibrinated undergoes a progressive decrease of CO_2-combining power on standing. These authors, Buckmaster (5), and Bohr (6) refer to changes of blood alkalinity induced by defibrination as indicated by the work of 20 to 40 years ago in which the blood was titrated. But it is not easy to say just how much bearing these early observations have on the present problem.

Experiment 4.—From a cannula in the femoral artery of a dog three blood samples of 10 cc. each were drawn in rapid succession. The first (*a*) was treated with ammonium oxalate under oil. The second (*b*) was carefully defibrinated by moderate stirring with a feather under oil. The third (*c*) was collected in a paraffined flask. All three were immediately equilibrated with 40 mm. of CO_2 at 37.5°C. The analyses of these samples showed

Sample (*a*) = 48 volumes per cent CO_2
" (*b*) = 54 " " " CO_2
" (*c*) = 48 " " " CO_2

Experiment 5.—Two samples of arterial blood were drawn. One was defibrinated, the other oxalated. Both were equilibrated with 40 mm. CO_2 at 37.5°C. for 2 hours. At intervals samples were withdrawn and analyzed. The results were as follows:

Time, *min*	15	30	60	120
Defibrinated blood, *vol. per cent*	54	52	46	42
Oxalated blood " " "	47	47	48	47

Experiment 6.—Oxalated arterial dog blood was allowed to stand exposed to the air at room temperature in a beaker. One sample of the original blood was equilibrated with air containing 40 mm. CO_2 and a second with air of 21 mm. CO_2. At intervals additional samples were taken from the beaker, and divided into two parts, of which one was immediately analyzed for CO_2 while the other was equilibrated with 40 mm. CO_2 and analyzed.

Time, *min*	0	60	120	180	240	300
CO_2 content, *vol. per cent*	41	37	33	30	25	21
CO_2-combining power at 21 mm.	30					
CO_2-combining power at 40 mm.	40	40	39	40	38	36

Evidently this blood underwent no fundamental change until, at the end of 180 minutes, its content of CO_2 fell below 30 volumes per cent and its tension below 21 mm.

Experiment 4 indicates that for the purposes of blood gas equilibration and analysis oxalated blood is much nearer to normal blood than is that which has been defibrinated.

Experiment 5 in turn indicates that even when defibrinated blood is kept at a CO_2 tension of normal amount it tends to undergo a progressive loss of CO_2-combining power. Oxalated blood on the contrary within any reasonable time does not exhibit this change.

In Experiment 6 it is to be seen, however, that if oxalated blood is exposed to air it likewise undergoes a progressive loss of CO_2-combining power when, but not until, the CO_2 tension falls below 21 mm. at room temperature—as shown by the results of analyses of samples separated at intervals and equilibrated again at 40 mm. of CO_2.

In Experiment 7 it will be seen that an even more rapid loss of CO_2-combining power may be produced in oxalated blood by passing through it a continuous stream of air free from CO_2. In the performance of this experiment difficulty was at first encountered because of the concentration of the blood by evaporation as indicated by determinations of total solids. In the experiment here given a closed circuit apparatus was employed. The blood was placed in a paraffined vessel immersed in a water bath at 37°C. and by means of a small blower air was circulated through this vessel and through a wash bottle containing a solution of sodium hydroxide.

Experiment 7.—Oxalated dog blood was subjected to a stream of CO_2-free air circulated as described in the text. The equilibrations and analyses were similar to those of Experiment 6.

Time, *min.*	0	30	45	60	120
CO_2 content, *vol. per cent*	52	10	10	8	9
CO_2-combining power at 40 mm	54	44		32	31

What the nature of the alteration in blood induced by overventilation may be we cannot as yet fully define. It appears, however, to be associated with several other phenomena among which the following may be mentioned.

Some of the corpuscles are ruptured and the plasma or saline is tinged with hemoglobin in solution, as is shown in Paper XI of this series.

Blood altered by overventilation does not thereafter meet the test for normality described in Paper VII of this series. Thus when two samples of such altered blood are exposed to different tensions of CO_2 (for instance, 40 and 50 mm.) and analyzed, and their plasmas are separated and analyzed, it is found that the plasmas do not show the same difference in CO_2 content as do the two samples of whole blood at the two tensions. This suggests that the alteration of the blood involves the development of abnormal differences of C_H between the fluid within the corpuscles and that surrounding them.

We are inclined to believe (but further work is needed) that the alteration induced by overventilation is also associated with the development in the corpuscles of an increased capacity for the interaction of oxygen and CO_2. Data bearing on this matter are given in the following paper.

CONCLUSIONS.

When blood is exposed to a tension of CO_2 below a critical level it undergoes an irreversible, or at least not readily reversible, change. Thereafter, if it is equilibrated with a normal tension of CO_2 it combines with distinctly less CO_2, and presumably produces correspondingly less $NaHCO_3$, than it did at this tension previously. Defibrinated blood is even more prone to such changes than oxalated blood, but oxalated blood likewise shows them.

Some associated phenomena are mentioned.

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HEMATO-RESPIRATORY FUNCTIONS.

X. THE VARIABILITY OF RECIPROCAL ACTION OF OXYGEN AND CO_2 IN BLOOD.

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(Received for publication, September 25, 1920.)

The opinion that variations of CO_2 content and of C_H in the blood influence the amount of oxygen absorbed or liberated by hemoglobin has won general acceptance.

If this view is correct, the converse is, on theoretical grounds, extremely probable; namely, that variations of oxygen tension and content influence likewise the capacity of the blood to hold CO_2.

Experiments directed by Ludwig (1) failed to show this effect, however; and Bohr (2) later had a similar negative result.

On the other hand Christiansen, Douglas, and Haldane (3) believe that they have proved the phenomenon; and L. J. Henderson (4) has recently based an extensive theoretical discussion upon their results.

We find, however, that the facts in the matter indicate much more complicated relations than any one has supposed. Thus the following experiments show that one and the same blood may have this property of gaseous reciprocity in marked degree, or may lack it, according to the way the blood has been treated. Our observations suggest that the same treatment which, as shown in Paper IX, induces a lowering of the CO_2 dissociation curve of the irreversible type also induces the capacity for reciprocal action of gases.

It is noteworthy that until recently, and to a large extent even now, most of the studies upon the blood gases have been made with defibrinated blood. In our hands, defibrinated blood commonly shows the property of interaction of oxygen and CO_2, while oxalated blood usually does not.

TABLE I.

Influence of Oxygen in Decreasing the CO_2 Capacity of Defibrinated Blood.

Sample No.	Equilibrating gas mixture.	CO_2 content of blood.		Average.
		vol. per cent		*vol. per cent*
1	Air + 40 mm. CO_2	53	52	52.5
	Hydrogen + 40 mm. CO_2	58	57	57.5
	Nitrogen + 40 " CO_2	58	57	57.5
2	Air + 40 mm. CO_2	50	48	49.0
	Hydrogen + 40 mm. CO_2	54	54	54.0
	Nitrogen + 40 " CO_2	55	54	54.5
3	Air + 40 mm. CO_2	48	46	47.0
	Oxygen + 40 mm. CO_2	47	44	45.5
	Hydrogen + 40 mm. CO_2	54	52	53.0
	Nitrogen + 40 " CO_2	52	53	52.5

For the experiments herewith tabulated fresh arterial dog blood was used. In some cases it was defibrinated; in others it was oxalated. In all cases the samples were equilibrated with the gas mixtures indicated in Tables I and II at a temperature of 40°C. and barometric pressures ranging between 755 and 765 mm.

TABLE II.

In Oxalated Blood the Presence or Absence of Oxygen Does Not Influence the Capacity for CO_2.

Sample No.	Equilibrating gas mixture.	CO_2 content of blood.						Average.
		vol. per cent						*vol. per cent*
1	Air + 40 mm. CO_2	42	43	42				42.3
	Nitrogen + 40 mm. CO_2	42	42	43	43			42.5
2	Air + 40 mm. CO_2	37	39	39	38	39	37	38.2
	Oxygen + 40 mm. CO_2	39	37	39				38.3
	Hydrogen + 40 mm. CO_2	39	36	38	39			38.0
	Nitrogen + 40 " CO_2	38	40	40				39.3
3	Air + 70 mm. CO_2	58	60					59
	Hydrogen + 70 mm. CO_2	56	58					57
	Nitrogen + 70 " CO_2	58	58					58

It will be seen that all the defibrinated bloods show the influence of oxygen in decreasing CO_2-combining power. With the oxalated bloods, on the contrary, none of the variations in the figures obtained lie beyond the range of analytical error.

CONCLUSIONS.

The action of oxygen upon the CO_2 capacity of blood is variable. It commonly occurs in dog blood which has been defibrinated, but not usually in that which has been oxalated. In the light of the results reported in previous papers bearing upon associated processes it appears to us probable that the distinction between the effects of the two methods of treating blood is in some way associated with the irreversible alteration discussed in Paper IX.

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HEMATO-RESPIRATORY FUNCTIONS.

XI. THE RELATION OF HEMOLYSIS TO ALTERATION OF THE H_2CO_3: $NaHCO_3$ EQUILIBRIUM.

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(Received for publication, September 25, 1920.)

In several of the previous papers of this series we have commented on the occurrence of hemolysis. From these observations it appears that there is a relation between hemolysis and the irreversible alteration of the blood discussed in Paper IX of this series.

When we first observed this relation we were inclined to think that the explanation might be that laked hemoglobin acts in plasma like an acid. Perhaps it does; but it now seems to us that the evidence on the whole indicates something more fundamental. One or the other, or an interaction of two processes may be involved. One is that excessive decrease of the load of HCl by exposure to abnormally low tensions of CO_2 may have effects within the corpuscles which include increased fragility. The other is that under conditions of abnormally low CO_2 ratio (relatively $H_2CO_3 < NaHCO$) and therefore low C_H there is a strain upon the surface of the corpuscles which tends to cause their rupture.

The matter is important as a possible hint regarding the physical chemistry of the hemolytic incompatibility of some bloods. It is, we think, quite certain that it affords the reason why plasma or serum obtained either by allowing blood to stand or by means of the centrifuge is often tinged with hemoglobin. Hemolysis usually occurs when the surface of the blood has been exposed to the air so that gaseous diffusion acts freely on even a thin layer; but the plasma is generally clear if the surface is protected with oil. In our experience the serum of defibrinated blood is generally

tinged with hemoglobin; and this, we believe, is largely due to the exposure of the blood.

In Table I are summarized the observations which occurred in experiments on the blood of a number of dogs. In each case a sample of arterial blood was oxalated, equilibrated with 40 mm. of CO_2, and analyzed. A second oxalated sample was equilib-

TABLE I.

Relation of Hemolysis to the Irreversible Alteration of CO_2-Combining Power Induced in Blood by a Preliminary Equilibration at an Abnormally Low Tension of CO_2.

Blood No.	First sample.		Second sample.		
	CO_2 content at 40 mm.	Hemolysis.	CO_2 content at 18 mm. and then at 40 mm.		Hemolysis.
	vol. per cent		*vol. per cent*		
1	45	−	32	44	−
2	41	−	25	39	+
3	33	−	24	30	+
4	36	−	20	26	++
5	30	−	21	29	−
6	30	−	19	30	−
7	39	−	26	36	+
8	37	−	24	31	++
9	31	−	23	29	+
10	33	−	20	33	−
11	28	−	20	28	−
12	27	−	19	27	−
13	40	−	32	36	+++
14	39	−	24	36	+
15	37	−	24	30	++
16	31	−	22	30	−
17	33	−	20	33	−
18	28	−	20	28	−
19	46	−	33	44	+
20	41	−	26	32	+

rated first with air containing only 18 mm. of CO_2, then with 40 mm. of CO_2, with analyses at each tension. As will be seen in the table, some degree of hemolysis was observed in more than half of the second samples but in none of the first. It is also to be seen in the table that the "irreversible effect" in reduction of CO_2-combining power occurred in exact parallelism to hemolysis.

Thus whenever the figure in Column 5 was distinctly lower than that in Column 2 hemolysis developed. Whenever the figures were virtually the same in these columns hemolysis was absent.

It is also to be seen in the table that hemolysis and the irreversible alteration of CO_2 capacity are not induced in all bloods at the same CO_2 tension or content. This is probably due to the fact that it is not only the HCl load of the corpuscles but also the previous alkali content of the plasma which determines the conditions, particularly the C_H, induced within and without the corpuscles by a low tension of CO_2 (for instance 18 mm.). If this view is correct, the data in the table suggest that by the methods used in Paper VIII of this series the resistance of the corpuscles to hemolysis of this type may be artificially increased or decreased. We hope to test this idea.

CONCLUSION.

The occurrence of hemolysis in connection with the irreversible alteration of CO_2 capacity induced by exposure of abnormally low tensions of CO_2 is demonstrated.

GASOMETRIC DETERMINATION OF NITROGEN AND ITS APPLICATION TO THE ESTIMATION OF THE NON-PROTEIN NITROGEN OF BLOOD.

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A number of reactions between sodium hypobromite and various nitrogenous substances are known in which nitrogen is liberated as a gas. Among this number are the reactions with urea, ammonium salts, uric acid, creatine, etc. A method for the quantitative estimation of the first named has been based upon the reaction in point, though it has now been discredited to a large extent. As far as the writer is aware the reaction between sodium hypobromite and ammonium salts has never been utilized in the quantitative determination of nitrogen. According to Krogh[1] this reaction falls short of being quantitative as in the case of urea. She found 97.5 per cent of the nitrogen of ammonium chloride to be liberated by sodium hypobromite and obtained a qualitative test for nitric acid in the solution after the reaction.

The experiments carried out heretofore have been performed at ordinary pressures. Some time ago it occurred to the writer that it would be of interest to carry out several of the above reactions in the apparatus devised by Van Slyke[2] for the determination of the carbon dioxide content of blood plasma; that is, *in vacuo*. The urea reaction was tried first and the outcome was clear-cut to the extent that this is possible with an apparatus of the capacity of that in question. Instead of a smaller quantity of nitrogen which is obtained when the reaction occurs at

[1] Krogh, M., *Z. physiol. Chem.*, 1913, lxxxiv, 379.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

atmospheric pressure, trial showed that the theoretical quantity of nitrogen is liberated. It was observed incidentally that the quantity of carbon dioxide produced is also in accordance with theory. Ammonium sulfate was then substituted for urea and again the theoretical quantity of nitrogen was obtained. Whether Krogh's results were due to the concentration or pressure relations obtaining in her experiments is not elucidated here, nor is the result in the case of the urea reaction, but the analyses submitted in this communication show that the yield of nitrogen obtained in the Van Slyke apparatus is quantitative. Inasmuch as in the Kjeldahl method for total nitrogen the first step is the conversion of the nitrogen of the sample into ammonium sulfate, we have in the hypobromite method an alternative procedure for the long distillation process of the Kjeldahl method, which is considerably shorter and requires no standard solutions. As described here, using the ordinary Van Slyke apparatus, the method does not have the accuracy obtainable by the Kjeldahl method. This is due to the impossibility of reading the volume of nitrogen with so high a degree of precision as one might wish. Enlarging the apparatus would help, but whether this is feasible the author has not attempted to decide. However, the accuracy obtainable with the ordinary apparatus is ample for many purposes.

The procedure is as follows. The sample is digested with as small a quantity of sulfuric acid as possible and a small crystal of copper sulfate. After cooling, it is diluted and the solution made up to some definite volume, for instance 100 cc. A definite quantity, preferably not more than 10 cc., is then placed in the apparatus. The dissolved air is liberated by subjection to a vacuum and expelled. Strong alkali sufficient to neutralize the sulfuric acid is run into the apparatus followed by 2 cc. of the hypobromite solution.[3] The pressure in the reaction chamber is reduced and after shaking for about a minute the liberation of nitrogen is complete. Apparently, if the hypobromite is present in more than a slight excess, there is some liberation of oxygen. In order to eliminate the error which would result from its pres-

[3] Two stock solutions are prepared. One contains 28 gm. of sodium hydroxide per 100 cc.; the other 12.5 gm. of sodium bromide and 12.5 gm. of bromine per 100 cc. For use one volume of each and three volumes of water are mixed.

ence, 1 cc. of a sodium pyrogallate solution (5 gm. of pyrogallate acid in 100 cc. of 28 per cent sodium hydroxide) is run into the reaction chamber as soon as the decomposition of ammonium sulfate is complete. After the oxygen has been absorbed the solution is freed from dissolved gases and is then lowered into the proper chamber whereupon the nitrogen volume is ready to be measured. This can be done so that the contained gas is at atmospheric pressure or by making the volume exactly 1 cc. and then measuring the pressure conditions necessary to accomplish this. A somewhat higher degree of accuracy appears to be obtainable by the latter procedure. It has the advantage that one is adjusting the meniscus at a set mark instead of estimating between marks, and, what is more important, one is always using the maximum capacity of the apparatus. Since the error of reading the volume is the same at any point, it is obvious that the error of any determination introduced by this cause will be smaller the greater the volume measured. On the other hand, if the quantity of gas is so small that the pressure must be reduced to 40 or 50 mm. Hg, for example, then small errors made in estimating the pressure introduce a relatively large error, though perhaps not so large then as when the volume is read at atmospheric pressure. Correction must be made for the nitrogen content of the hypobromite solution. This is approximately 0.009 cc. (at 760 mm. Hg and 0°) per 1 cc. of solution. The nitrogen content of the strong alkali and pyrogallate is negligible.

The quantity of nitrogen dissolved in the final solution is worthy of consideration. If the quantity of solution amounted to 10 cc. and the amount of nitrogen liberated was about 1 cc., then, since the capacity of the apparatus is 50 cc., the minimum nitrogen pressure obtainable in the apparatus would be $\frac{1}{4}\frac{1}{0}$ of an atmosphere. 1 cc. of water dissolves 0.01639 cc. of nitrogen at 20° and 760 mm. pressure. At $\frac{1}{4}\frac{1}{0}$ of an atmosphere the amount would be $\frac{1}{4}\frac{1}{0} \times 0.01639 = 0.00041$ cc. per 1 cc. of water, or 0.0041 cc. per 10 cc. The solvent here is not pure water, however, but a rather strong solution of sodium sulfate, sodium bromide, and sodium pyrogallate in which the nitrogen solubility is considerably less than in pure water. The exact concentration is a variable quantity and of course no solubility data exist which meet the present demand. If it is assumed that a 10 per cent solution

of sodium sulfate is approximately correct, then the correction per 10 cc. would be about 0.002 instead of 0.0041 cc. This may be 50 per cent from the true value without introducing an error greater than 0.1 per cent into the final result. If the volume of gas measured is 0.5 cc. instead of 1 cc., then the correction just suggested is halved. According to the accuracy desired the solubility factor may be taken into account or neglected.

The results obtained on various substances are given in Table I.

TABLE I.

Substance.	Found.	Theory or Kjeldahl
	cc.	cc.
Sodium sulfanilate	5.92	6.07
" "	6.04	6.07
" "	6.15	6.07
" "	6.15	6.07
" "	6.15	6.07
Casein	13.6	13.9
"	13.7	13.9
"	13.8	13.9
"	13.8	13.9
"	13.8	13.9
Uric acid	32.5	32.4
" "	32.7	32.4
" "	32.8	32.4
" "	33.6	32.4
Dried milk	5.37	5.35
" "	5.42	5.35
Urea	46.5	46.7
"	46.7	46.7

The method for the determination of total nitrogen just described may be applied to the determination of the non-protein nitrogen of blood. The tungstic acid precipitation method described by Folin and Wu[4] for obtaining a protein-free blood filtrate is well adapted for the present method. 5 or 10 cc. of the filtrate are digested with 0.5 cc. of nitrogen-free sulfuric acid and 1 drop of a 5 per cent copper sulfate solution. Small Kjeldahl flasks (30 cc.), with necks bent almost at right angles about 1½ inches from the end, serve well for the digestion. They are heated

[4] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

in a Bunsen flame before use to reduce bumping. If the heat from the microburner used in the digestion is controlled by hand manipulation, there is little danger of loss of material. After the contents of the flask have charred, the burner may be set under the flask with the flame reduced. At the first sign of an ominous stillness in the flask a piece of quartz sand is dropped in, and active though gentle boiling immediately occurs. This process is repeated as often as necessary until the digestion is complete. Digesting with the sulfuric-phosphoric acid mixture recommended by Folin and Wu hastens the process, but the method has disadvantages which outweigh the 2 or 3 minutes saved. After cooling the digest, it is diluted with 4 or 5 cc. of water and the sulfuric acid is partially neutralized with strong alkali. This is done merely to save the generation of unnecessary heat in the apparatus. One can determine how much strong alkali (55 per cent) is required for 0.5 cc. of sulfuric acid, and add about two-thirds of this quantity. The solution is again cooled and then transferred to the apparatus. The volume of liquid should be about 10 cc. (It is desirable, of course, to keep the volume as small as possible but if sufficient water is not present sodium sulfate crystals will form.) Dissolved air is then eliminated in the usual manner. If this is not well attended to a relatively large error will be introduced in the case of a blood normal with respect to its quantity of non-protein nitrogen inasmuch as the amount of gas liberated is small. Sufficient alkali to complete the neutralization of the sulfuric acid is now run into the vacuum chamber followed by 1 cc. of the hypobromite solution described. The apparatus is immediately evacuated and shaken for about a minute. As soon as the reaction is complete 1 cc. of pyrogallate solution is admitted and the apparatus is again shaken to insure the absorption of any oxygen. As a rule the heat of neutralization is sufficient to warm the apparatus several degrees. It is necessary, therefore, to allow it to stand for a time before reading the volume of nitrogen or it may be immersed in a cylinder of water for a few minutes. After the gas is at room temperature the volume is measured in the usual manner. If the blood were a normal specimen the volume of gas obtained from 5 cc. of filtrate would be in the neighborhood of 0.20 cc. The device of making the volume 1 cc. arbitrarily is

appropriate here. In reducing the volume to standard conditions substitution in the ordinary gas formula is suitable for an occasional determination, but for routine work the tables given in Landolt-Börnstein-Roth's "Tabellen" are a great convenience.

TABLE II.

Non-Protein Nitrogen, per 100 Cc. Blood.

Hypobromite method.	Kjeldahl method.
mg.	*mg.*
39.5	41.8
39.3	39.3
38.7	36.0
38.3	41.9

Results are easily duplicated by the method just described and to some who have difficulties with colorimetric methods the choice of a method based upon a perfectly clear-cut chemical reaction may be welcome. The results have been checked by the Kjeldahl method (Table II), though this could hardly be considered essential in view of the results given in Table I.

RELATION OF FODDER TO THE ANTISCORBUTIC POTENCY AND SALT CONTENT OF MILK.*

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In 1914 Funk (1) raised the question as to whether milk varies in its vitamine content according to the fodder of the cow. Since this time several other investigators have referred to an interrelationship of this nature, notably Barnes and Hume (2) in a recent paper on the antiscorbutic content of milk. In an article treating of the fat-soluble vitamine, we suggested an insufficiency of this dietary factor in milk as the result of the winter fodder of cows (3). Indeed, in view of the probability that the animal organism is incapable of synthesizing vitamines, and is dependent on the food for its supply, one should expect the milk to be greatly affected by the dietary intake. The first experimental proof of the existence of this interrelationship has been furnished recently by the work of Hart, Steenbock, and Ellis (4). These investigators conclusively show that milk secreted by cows receiving dry fodder contains far less antiscorbutic vitamine than milk secreted by cows on summer pasture. The herd which they tested had never been given any "fresh green vegetable tissue," having been fed continuously on "air-dried roughages and grains." The summer pasture milk was derived from cows which grazed part of the day on grass and clover.

Our investigation, which was undertaken last spring, differs from that of Hart, Steenbock, and Ellis mainly in a delimitation of the duration of the feeding periods, and in the inclusion of a chemical examination of the two varieties of milk. The plan of the experiment was as follows: Five grade Holstein cows which had been freshened about 2 months previously were employed

* Presented before the Society of Experimental Biology and Medicine, New York, October 20, 1920.

for the entire test. These cows had been stall-fed throughout the winter on a normal winter ration of ensilage, hay, and concentrates. Beginning April 30th, 1920, and for 21 days thereafter, the cows were fed on a daily ration of 25 pounds of a concentrate mixture which consisted of one part of bean meal, two parts of oil meal (flaxseed extracted by pressure), two parts of hominy, two parts of gluten meal, and two parts of bran; they received also 8 pounds of kiln-dried beet pulp, 4 quarts of molasses, and 12 pounds of straw. The object of this dietary was to provide a food which was ample in all respects excepting in its quota of antiscorbutic vitamine. For the first few days the cows manifested a marked loss of appetite, but thereafter they consumed practically the entire amount of the dry ration. They had produced, prior to this experimental feeding, an average of about 40 pounds of milk per day. At first this change in fodder resulted in an average decrease in milk production of about 10 pounds per cow per day, but later the animals became more accustomed to the ration, so that at the end of the 21 day period there was an average decrease in milk flow of only 5 pounds per day. The general health of the cows had not been maintained. After 3 weeks of this antiscorbutic-free ration a mixed sample of milk from all five cows was collected and dried by means of the Just roller process, by which it is subjected to about 230° F. for a few seconds.

The cows were then placed on pasture for 3 weeks, a small amount of concentrate being given, in addition, for the first few days only. After 3 weeks pasturage, a day's milking of the five cows was collected and dried. Throughout this second period, excepting for the first few days, the cows received only fresh grass. The physical condition of three of the cows showed marked improvement, but two did not respond so quickly to the change in fodder. The production of milk did not increase materially during this period of pasturage, and at its close was still 5 pounds less per cow per day than before the concentrated feeding had been instituted.

The object of using dried rather than fluid milk for this experiment was that it permitted a test of both varieties of milk to be carried out synchronously and under identical conditions. It is evident that only by this means could dry fodder milk and pas-

ture milk from the same cows be tested at one and the same time. Furthermore, it made it possible to define sharply a feeding period—3 weeks—and thereby to judge the effect on the quality of the milk of a dietary given during this limited period. Unless dried milk were used, the pasture milk would represent a product varying according to the ever lengthening time that the cows had been grazing, whereas by using dried milk the entire quantity was obtained from 1 day's milking just 3 weeks after dry fodder had been discontinued.

The milk was kept tightly sealed in tin containers until required, and was then diluted with water to its original water content. Guinea pigs were fed the equivalent of 80 cc. *per capita* daily of fluid milk, in addition to oats and bran. This amount of milk was selected because previous tests with dried milk had shown that it contains what may be termed the borderline protective dose of antiscorbutic vitamine, and that when guinea pigs are given more than this quantity they fail to develop scurvy. Every animal received its full quota daily, supplementary hand feeding being resorted to when necessary.

Charts 1 and 2 illustrate the results of two sets of feeding tests. It is seen that all the guinea pigs fed with the dry fodder milk developed scurvy within a period of 21 days, and died of scurvy within 56 days. On the other hand, the animals which received pasture milk are still alive after more than twice this interval—120 days—two showing mild signs of scurvy. The contrast is striking, and definitely illustrates the marked difference in antiscorbutic content of the two varieties of milk.

When we bear in mind that this marked alteration in the quality of the milk took place in cows which had been on pasture but 3 weeks, it is evident that the antiscorbutic vitamine of the food is rapidly secreted into the milk by the lacteal glands, even in the extreme instance where the body has been absolutely starved of this vitamine. The tissues do not seem to replenish their deficiency of this vitamine or to hoard it before excreting any from the body by way of the milk. This result bears out some unpublished experiments which showed that guinea pigs are unable to store the antiscorbutic vitamine, that even though their diet has contained it in large excess such provision does not protect them from a subsequent period of antiscorbutic vita-

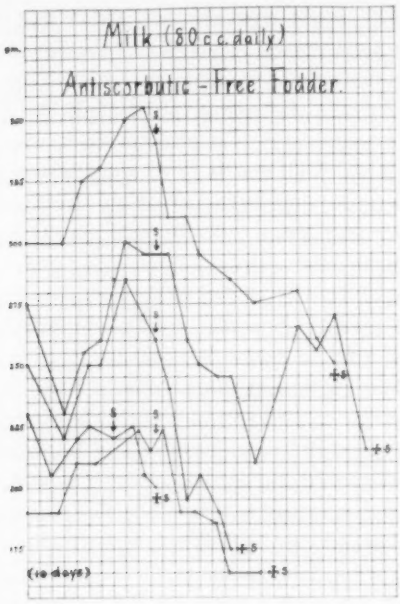

CHART 1. Guinea pig feeding experiment. The cows producing this milk had been fed on fodder containing almost no antiscorbutic vitamine for a period of 3 weeks.

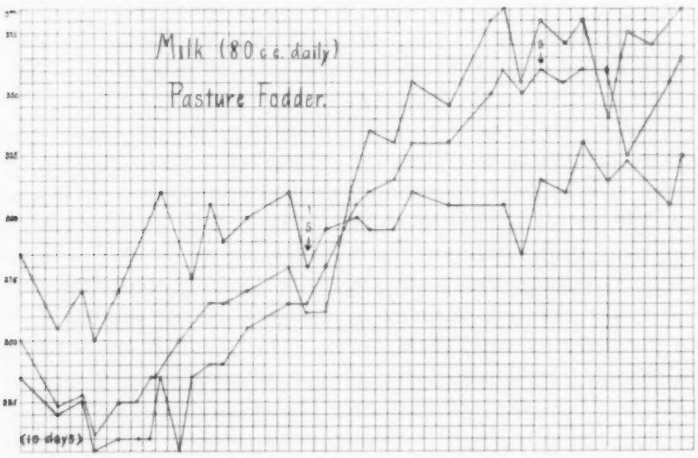

CHART 2. Guinea pig feeding experiment. Milk from the same cows as in Chart 1 after they had been pasture-fed for a period of 3 weeks.

mine starvation. In other words, these animals seem to be carrying on a precarious hand-to-mouth existence in relation to this essential dietary factor.

The feeding test with dry fodder milk demonstrates that it contained but little antiscorbutic vitamine. If we compare this result with that obtained when guinea pigs are fed an equivalent amount of milk which has been autoclaved at 110°C. for 1 hour in order to destroy its antiscorbutic vitamine, we find that there is little difference in the length of time it takes for scurvy to develop; the sole advantage enjoyed by the guinea pigs fed on the dry fodder milk is a somewhat longer duration of life. Such a result must be interpreted as indicating that at the end of the first stage of our test the cows were secreting milk which was almost devoid of the antiscorbutic vitamine, and that when the supply of this dietary factor is entirely cut off, the milk is rapidly depleted of its antiscorbutic content. It should be remembered, however, that the feeding conditions were exceptionally rigid, so severe as to be rarely encountered under natural circumstances in man or in animal.

Table I shows the composition of the milk as affected by the two kinds of fodder. A composite sample of the liquid milk from all five cows was used for the analyses, with the exception of the lecithin determination, for which dried milk was employed. A study of the table shows that the fat, protein, and sugar percentages were very similar, but that there were certain well defined differences in the composition of the ash of the two milks. Passing over minor variations, it is seen that the percentages of calcium and of phosphorus were significantly higher in the pasture milk and that its citric acid content was over 50 per cent greater.[1] On the other hand there was less sulfur in this milk, in spite of its somewhat higher protein content.

The percentages of calcium and of phosphorus in the antiscorbutic-free milk are below the normal; those of the pasture milk are within normal limits. The question of a causal relationship naturally suggested itself between the lack of antiscorbutic vitamine and the decreased excretion of these salts

[1] Citric acid was determined by the Bacon and Dunbar method (Bacon, F., and Dunbar, P. B., *U. S. Dept. Agric., Bureau of Chemistry, Circular 78*, 1911).

into the milk. A conclusive answer to this question must be postponed, however, until further data are available.

The increase in citric acid may be explained in part by the greater amount of citrates contained in the green fodder, but gains added interest in view of the close association between citric acid and many of the most potent antiscorbutic foodstuffs. In view of the marked variation of this constituent in the two

TABLE I.

Composition of Milk as Affected by Fodder.

	Antiscorbutic-free fodder.	Pasture.
	per cent	*per cent*
Water	88.38	88.19
Total solids	11.62	11.81
Fat	3.37	3.44
Total proteins (N × 6.38)	2.82	2.93
Casein (N × 6.38)	2.28	2.32
Albumin (N × 6.38)	0.42	0.49
Extractive nitrogen	0.02	0.02
Lecithin	0.069	0.7
Lactose	4.73	4.56
Citric acid	0.08	**0.13**
Ash	0.606	0.670
Potassium oxide	0.150	0.157
Sodium "	0.051	0.056
Calcium "	0.138	**0.165**
Magnesium "	0.009	0.005
Iron "	0.0001	0.0002
Phosphorus pentoxide	0.158	**0.190**
Sulfur trioxide	**0.023**	0.014
Chlorine	0.054	0.097

varieties of milk, normal figures which have been established without regard to the fodder of the cows must be regarded as incomplete (5). In fact a lesson to be derived from this chemical examination of antiscorbutic-free and of pasture milk is the danger of setting up rigid standards for milk constituents, and the necessity of prefacing such results with full data as to the diet of the lactating animal.

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CREATININE AND CREATINE IN THE BLOOD.

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Few figures have been published for the creatinine and creatine in the blood of normal women. Hunter and Campbell (1) make the tentative suggestion that blood creatinine is lower for women than for men, but all the women they observed were either in late stages of pregnancy or were convalescent after childbirth, and were therefore very inactive. They also conclude that the creatinine is "lower in subjects deprived of exercise than in those leading an active life." It may be, therefore, as they themselves suggest, that the inactivity rather than the sex of the subjects is the cause of the difference. Their average plasma creatinine for forty-three males is 1.04 mg. per 100 cc. and for seventeen females 0.87 mg. Plass (2) also gives lower figures for creatinine concentrations in women than in men—1.0 to 1.2 mg. and 1.3 to 1.5 mg. per 100 cc. respectively—but he does not give a detailed report on this investigation.

Creatine, on the other hand, according to Hunter and Campbell (1) seems to be higher in the blood of females than of males, 6.81 mg. on the average (uncorrected) as opposed to 5.57 mg. per 100 cc. It is also higher in women in late stages of pregnancy than in normal women (3).

Considerable variation in creatine is to be expected both from individual to individual and in the same individual from day to day. The creatinine is a much less variable quantity. Hammett's (4) recent series of weekly analyses of blood from the same individuals shows this difference plainly. He divides the various blood constituents into three groups according to the amount of variation from day to day in the same individual. Creatinine is in the first group, the least variable of all; and creatine in the

second group, more variable than creatinine, total non-protein nitrogen, and total nitrogen, and less variable than uric acid, amino-acid, and urea nitrogen. Some of Hammett's subjects were women but he does not say which figures belong to the women and which to men.

TABLE I.

Experimenters.	Creatinine per 100 cc.	Creatine per 100 cc.
	mg.	*mg.*
Folin and Denis (5)	1.1 –1.4	5.2 – 8.4
Myers and Fine (6)	1.0 –2.0	5.0 –10.0
Gettler and Baker (7)	0.1 –0.5	3.0 – 6.5
Feigl (8, 9)	< 1.0 > 2.5	
Hunter and Campbell (1)	0.7 –1.3	2.7 – 3.5 Average about 3.0.
Pemberton and Buckmann (10)	0.6 –1.3	3.7 – 5.0
Hammett (4)	1.08–1.67	3.00– 6.21

It is not surprising, therefore, that the many published figures for creatinine should be in fairly close agreement (with the exception of those of Gettler and Baker (7)) and that the creatine figures should show considerable variation. Also the older method for creatine was much more faulty than that for creatinine. Table I summarizes most of the published creatinine and creatine figures, the great majority of them being for men.

No work has been done on variation of creatinine and creatine in blood during menstruation. That such variation might be expected could be anticipated by the work of Krause (11) and that of Rose (12) on urine. The results of the former show that on a creatine- and creatinine-free diet creatine is invariably present in the urine after menstruation, while the latter states that the creatine in urine tended to disappear during menstruation in a number of the cases studied, though in most cases no regularity was observed.

It therefore seemed desirable to obtain more figures on the blood creatinine and creatine of normal women and especially to determine whether there is any regular variation during menstruation.

EXPERIMENTAL.

For our experiments we had as subjects twenty-four apparently normal women students or faculty members ranging in ages from 20 to 41 years and following the ordinary University regime with a good deal of laboratory work. They were, therefore, decidedly more active than those who served Hunter and Campbell as subjects.

TABLE II.

Intermenstrual and Menstrual Creatinine and Creatine per 100 Cc. of Blood.

Subject.	Age.	Preformed creatinine.		Creatine as creatinine.	
		Intermenstrual.	Menstrual.	Intermenstrual.	Menstrual.
	yrs.	*mg.*	*mg.*	*mg.*	*mg.*
E. V.	20	1.17	1.58	4.65	3.77
Z. T. W.*	21	0.96	1.20	3.45	3.22
B. W.	21	1.38	1.10	4.05	2.68
H. K.	22	1.36		2.23	
E. M.	22		1.42		2.56
B. W.	24	1.10		3.30	
P. R.	24	1.62	1.55	2.81	2.89
M. A. P.†	26	1.22	1.11	2.90	3.38
W. S.	26	1.10	1.13	2.80	2.45
L. M. D.†	28		1.37		3.34
M. O. L.	28	1.00	1.38	2.28	2.35
R. M.	28	1.33	1.33	3.08	2.99
G. B.	28	1.07	1.65	3.22	3.29
A. N.	28	1.18	1.36	3.20	3.14
S. W.	29		1.46		3.12
J. C.	33	1.39	1.36	3.35	4.01
M. M.	34	1.56	1.02	3.07	2.64
L. K.	36	1.27		3.23	
L. K.	36	1.01		4.52	
L. D.	37	1.27	1.35	2.80	3.19
R. L. R.	40	1.39	1.50	3.12	4.09
F. R.	41	1.28	1.34	3.47	3.74
Average		1.25	1.35	3.24	3.16

* Menstrual specimen 2nd day of period.

† Irregular menstrually; 3 weeks overdue.

The blood specimens were drawn on the first day of the menstrual period, if possible, but in a few instances on the second day (exceptions which are listed in Table II), and a second speci-

men during the intermenstrual period. No definite regularity as to the hour for drawing the samples was followed but in all but a very few cases the time was between 11 a.m. and 12 m. or late in the afternoon; that is, 3 or 4 hours after the last meal. The diet of the subjects was not controlled, but their general habit was to live on a rather low protein level.

In determining the creatinine and creatine Folin and Wu's (13) new method was exactly followed. The picric acid was purified according to the method of Folin and Doisy (3). The creatinine for the standard, obtained from the Central Scientific Company, was tested for purity by recrystallization from alcohol and comparison of the recrystallized and original substances in the colorimeter. No difference was observed.

The results of the analyses are given in Table II.

Discussion of Results.

We find no regular variation in the creatinine and creatine during menstruation. The average of all results for creatinine for the intermenstrual period is 1.25 mg., while for the menstrual period the average is 1.35 mg., an increase of only 7.4 per cent. For the creatine, we find an average of 3.24 mg. during the intermenstrual period and of 3.16 mg. during menstruation, which is a decrease of 2.5 per cent.

Of the fifteen individual cases with both menstrual and intermenstrual observations, nine show higher creatinine during the menstrual period, five lower, and one no difference. The greatest difference amounts to 0.58 mg. For creatine, seven show lower figures during menstruation and eight higher.

Thus, while the figures seem to show an occasional tendency toward increased creatinine and decreased creatine during menstruation, the proportion of the cases showing this tendency is not high enough to justify any definite conclusion. It is safer to conclude that the variation is not regular and is no greater than expected from day to day.

It is of interest to compare our figures with those of other investigators, summarized in Table I. Our ranges for creatinine, 0.96 to 1.65 mg., and for creatine, 2.23 to 4.65 mg., are within the limits of other investigators. All but five of our creatinine results fall within the limits set by Hammett (4). Our creatine

results are lower than his. Thirteen out of thirty-seven of our figures are below his lower limit and none reaches his upper limit, 6.21 mg.

Our results thus fail to substantiate the suggestion of Hunter and Campbell (1), that the creatinine appears in lower concentration in the blood of women than of men.

Like Hunter and Campbell (1) we fail to find figures in agreement with work by Feigl (9) indicating an increase of creatinine with age. Feigl worked with a large group of people ranging in age from under 10 years up to 80 years. We have only a small number falling in any of the three decades represented, and it is impossible to draw any definite conclusion that there is a greater concentration with increased age.

Influence of Sugar Diet on Creatinine and Creatine Determination.

We also had as a secondary problem the influence of sugar on blood creatinine and creatine. We made analyses on the blood of ten women on a normal diet, usually from 1 to 2 hours after a meal, and, again on a different day, 1 hour after the subject had eaten 70 gm. of sugar. Table III shows the comparative results.

TABLE III.

Results on High Sugar Diet per 100 Cc. of Blood.

Subject.	Age.	Preformed creatinine.		Creatine as creatinine.	
		Low sugar.	High sugar.	Low sugar.	High sugar.
	yrs.	*mg.*	*mg.*	*mg.*	*mg.*
B. W.	24	1.10	1.41	3.30	4.25
J. C.	33	1.39	1.59	3.35	3.67
H. K.	22	1.36	1.21	2.23	3.40
A. N.*	28	1.18	1.57	3.20	
L. K.	36	1.01	1.28	4.52	3.80
L. D.	37	1.27	1.54	2.80	2.78
E. V.	20	1.17	1.49	4.65	4.55
W. S.†	26	1.10	1.31	2.80	2.40
E. D.	22	1.48	1.56	3.81	3.72
L. G.†	22	1.03	1.10	3.45	4.36
Average		1.21	1.41	3.41	3.66

* High sugar taken on 2nd day of menstruation.
† " " " " 1st " " "

Discussion of Table III.

On the high sugar, with one exception, the creatinine was higher than without sugar. The greatest increase is 0.39 mg., and the least 0.07 mg. The average for all shows 1.21 mg. of creatinine on the normal diet and 1.41 mg. on the high sugar; an increase of 0.20 mg. or 17 per cent.

The creatine readings were not so noticeably influenced, probably because the possible increase due to glucose was masked by the usual day by day variation. The average without the sugar was 3.41 mg. and with the sugar was 3.66 mg.

SUMMARY AND CONCLUSIONS.

1. Observations were made on the creatinine and creatine of the blood of twenty-four normal women, fifteen of them being observed during both intermenstrual and menstrual periods.

2. No regular variation was found during menstruation for either creatinine or creatine.

3. In nine cases there was a slight creatinine increase during menstruation and in five a decrease. On the other hand, seven cases showed lower creatine during menstruation and eight higher.

4. The range for creatinine in the different subjects was from 0.96 to 1.65 mg. per 100 cc. The average content was found to be 1.30 mg. This falls within the range observed by other investigators, chiefly on men, and fails to substantiate the suggestion that blood creatinine is lower in women.

5. The range for creatine concentration is from 2.23 to 4.65 mg. per 100 cc., which is slightly lower than the range observed by other investigators.

6. No relation was observed between the age of the subject and increased creatinine.

7. The accuracy of creatinine determinations may be affected by high sugar diet, through the additional development of color by glucose.

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